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Neuroendocrine Implications for Coping and Alcohol Consumption in Response to Exercise Restriction

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NEUROENDOCRINE IMPLICATIONS FOR COPING AND ALCOHOL CONSUMPTION IN RESPONSE TO EXERCISE RESTRICTION

By

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ACTH = adrenocorticotropic hormone

ANOVA = analysis of variance

AUD = alcohol use disorder

B6 = wildtype C57BL/6J mouse

βE-HT = β-endorphin heterozygote

βE-KO = β-endorphin knock out

BEC = blood ethanol content

BNST = bed nucleus of the stria terminalis

CAST = castrated

CORT = cortisol (humans), corticosterone (animals)

CRH = corticotropin-releasing hormone

CRH-R1 = corticotropin-releasing hormone receptor 1

DHT = dihydrotestosterone

dmPFC = dorsomedial prefrontal cortex

DSM = Diagnostic and Statistical Manual of Mental Disorders

EDTA = Ethylenediaminetetraacetic acid

ELISA = enzyme-linked immunosorbent assay

ER = estrogen receptor

EtOH = ethanol

FSH = follicle stimulating hormone

GABA = gamma-aminobutyric acid

GAPDH = glyceraldehyde-3-phosphate dehydrogenase

GnRH = gonadotropin-releasing hormone

HPA axis = hypothalamic-pituitary-adrenal axis

HPG axis = hypothalamic-pituitary-gonadal axis

LC = locus coeruleus

LH = leutenizing hormone

mRNA = messenger ribonucleic acid

OVX = ovariectomized

PTSD = posttraumatic stress disorder

PVN = paraventricular nucleus (of the hypothalamus)

SEM = standard error of the mean

VH = ventral hippocampus

qPCR = quantitative real-time polymerase chain reaction

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ABSTRACT

The purpose of our study is to investigate the role that gonadal hormones and β-endorphin play in alcohol consumption in response to exercise restriction in a mouse model. Men and women consume alcohol in different manners from each other, both in terms of general consumption and in the factors motivating consumption. The murine model of alcohol self-administration that we utilize in this study represents both facets of this sex difference; female mice consume more alcohol than male mice and also are more likely to significantly increase their consumption when restricted from voluntarily accessing running wheels.

We hypothesized in our first experiment that if activational effects of ovarian hormones promote elevated vulnerability to exercise-restricted alcohol consumption, then ovariectomy would reduce the propensity to drink more alcohol when running wheels were unavailable. Our next hypothesis was that if activational effects of testicular hormones protect against exercise restriction-induced alcohol consumption, then castration would enhance alcohol consumption in response to wheel restriction compared to controls. To test these hypotheses, 47 female and 33 male mice underwent gonadectomy surgery, sham surgery, or were surgery-naïve. Using a limited access paradigm where animals had access to 20% ethanol solution for two hours each day, we measured alcohol consumption when mice had voluntary access to a running wheel, compared consumption when exercise-restriction stress was imposed by restricting access to the running wheels. We found no effects of either ovarian hormones or testicular hormones on alcohol consumption in response to exercise restriction, although

ovariectomy did decrease overall alcohol consumption, consistent with ovarian hormones mediating the tendency for females to self-administer higher levels of alcohol overall.

Our second set of studies examined the effects of β-endorphin on exercise restriction-induced alcohol consumption. β-endorphin negatively regulates the stress response, is released in response to alcohol consumption, and depleted levels of the hormone have been clinically correlated with elevated risk for excessive alcohol consumption. We hypothesized that effective self-medication with alcohol is dependent on β-endorphin inhibition of the stress axis. This may be implicated in the observed escalation in alcohol self-administration in response to exercise restriction in females and may interact with testicular hormones in male mice. To test these hypotheses, transgenic mice with varying levels of β-endorphin were subjected to the exercise-restriction self-administration paradigm. The male mice in these experiments were also subjected to either castration or sham surgeries to facilitate an understanding of the interaction between testicular hormones and β-endorphin levels. In females, low β-endorphin conferred enhanced sensitivity to drinking in response to wheel restriction, but this effect was not present in male subjects, regardless of gonadal condition. Female mice also displayed genotypic differences in plasma corticosterone levels and corticotropin-releasing hormone mRNA content in stress-related brain regions, suggesting alterations in stress-response activity as a function of β-endorphin levels.

Together these results suggest that activational effects of gonadal hormones do not explain observed sex differences in exercise restriction-induced alcohol self-administration; and β-endorphin deficiency confers elevated risk for exercise restriction-

induced alcohol consumption in a manner that does not interact with testicular hormones. With further investigation, the data presented in this thesis could assist in the development of individualized treatment and prevention plans in alcohol use disorder.

INTRODUCTION

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I. Introduction to Alcohol Use Disorder

Addiction is defined by the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) as "A maladaptive pattern of substance use leading to clinically significant impairment or distress" and is classified based on the severity of detriment to everyday life, as well as the degree of physiological adaptation to the substance of abuse (American Psychological Association, 2013). Using a slightly older edition of the DSM criteria developed in 1987 (DSM III R), lifetime prevalence of any alcohol use disorder was estimated to be as high as 32% of the population (Hasin, Stinson, Ogburn, & Grant, 2007). Alcohol use disorders (AUDs) cause an immense amount of strain on individuals,

families, and society each year. Estimated annual costs of AUDs in the United States exceed $200 billion when considering health care, loss of productivity, and incarceration costs (Bouchery, Harwood, Sacks, Simon, & Brewer, 2011). All alcohol consumption, interchangeably referred to as ethanol (EtOH) consumption, whether chronic or infrequent, only results in AUD development a portion of the time. Understanding what puts some individuals at an elevated risk for developing an AUD is a very pressing question, but it is a question to which we do not yet have the answer.

Individual differences and sex differences affect not only the consequences of drinking, but also impact why someone chooses to consume alcohol in the first place. Some are motivated by the positive reinforcing effects of alcohol: the addition of a reinforcer such as euphoria. Others are motivated by the negative reinforcing effects of alcohol: the removal of an aversive state such as social anxiety or stress. Relief drinking, or drinking to alleviate states of negative emotionality have been cited as critical motivators of alcohol consumption from very early in alcohol research (Jellinek, 1960; Wikler, 1948). Transition to addiction involves a shift from positive reinforcement as the primary motivating factor behind consumption to primarily negative reinforcement. Because the body and brain aim to neutralize and compensate for the effects of drugs, negative reinforcement in an addicted state often comes from alleviating a state that is itself brought on by chronic drug use. For example, chronic alcohol consumption results in depressed dopamine levels, which can be restored to normal levels by consuming alcohol (Volkow et al., 2007).

Men and women experience AUDs and subsequent recovery processes differently from each other. Women are more likely to experience persistent consequences after having an alcohol use disorder and are also more prone to developing alcohol-related physical conditions, including alcohol-related liver disease, cardiomyopathy, and breast cancer. Even after ostensible recovery from an AUD has been completed, women are more likely to report continued AUD symptoms, such as heightened levels of substance use, antisocial behavior, and social dysfunction (Agabio, Pisanu, Gessa, & Franconi, 2016; Foster, Hicks, Iacono, & McGue, 2014). Patterns of female consumption are sometimes complicating factors to understanding the prevalence of AUDs and their effects in women. Women tend to consume alcohol in private and in lower quantities, causing them to be a less visible group to include in formal alcohol studies (Fernandez-Guasti, Fiedler, Herrera, & Handa, 2014; Foster, Hicks, Iacono, & McGue, 2015; Greenfield, Back, Lawso, Brady, 2010).

The availability of treatment options is directly related to our ability to understand the factors driving AUDs. Current treatment options are relatively limited and are not equally sought by all addicts (Appell, Ellison, Jansky, & Oldak, 2004; Johnson, Cloninger, Roache, Bordnick, & Ruiz, 2000; Marsh & Miller, 1985). Women are one such subgroup of the population that are less likely to enroll in formal treatment programs. Of the limited treatments, few are pharmacological in nature, suggesting deficits in our understanding of the mechanisms of addiction. When women do seek treatment, many pharmacological interventions are less efficacious because drug development research has historically been conducted in male-only cohorts (Agabio et

al., 2016; Greenfield et al., 2010). The matters of differential efficacy based on sex as well as the need for unique doses of drug administration in males and females have been overlooked in many situations, to the detriment of women's health.

II. Sex Differences in Alcohol Consumption

AUDs are assumed in our society to affect men much more often than women, but the gender gap that has historically existed in addiction of all classes is decreasing. Today the male:female ratio is close to 3:1, significantly higher than previously reported ratios (Greenfield et al., 2010). The proportion of female adolescents with AUD is rapidly increasing and women demonstrated a faster progression between first alcohol consumption and the onset of significant alcohol-related problems and treatment entry, a process known as telescoping (Agabio et al., 2016; Hernandez-Avila, Rounsaville, & Kranzler, 2004). Women also suffer from stress-related psychiatric disorders at about double the rate of men, necessitating an understanding of the interaction between stress-related experiences and alcohol use (Kessler, McGonagle, Swartz, Blazer, & Nelson, 1993; Marcus, et al., 2005).

Because men and women are susceptible to psychiatric conditions, including anxiety and stress in different ways, they also have differential likelihood to engage in alcohol consumption when under aversive or stressful situations. Women commonly deal with stressful events through internalizing and men through externalizing coping behaviors (Foster, et al., 2015). Internalizing behaviors are focused inward and include

social withdrawal and fearfulness, and externalizing behaviors are directed outward and frequently involve violence. Fernandez-Guasti and colleagues observe that states of negative emotionality like chronic stress, low sense of mastery, and rumination are all more common in women than in men (Fernandez-Guasti, et al., 2012). The accumulation of stress in women, including post-traumatic stress disorder (PTSD), independently puts them at elevated risk for AUD risk compared to men, even if men accumulate more overall psychosocial risk factors (Foster et al., 2015; Sartor et al., 2010). This likely indicates that for women, the negative reinforcement of alcohol through alleviation of negative emotionality states is therefore a common motivator.

III. The Stress Response

Stress and the ability to cope with stressful situations have been implicated as causal factors in the development of alcoholism (Bolton, Cox, Clara, & Sareen, 2006; Brown, Vik, Patterson, Grant, & Schuckit, 1995; Gianoulakis, et al., 1989). Understanding the role of stress in alcohol use is an immense task, partially because the definition of stress is so ambiguous. Stress can be defined as the body's physiological response to a stressor, including the activation of stress-related systems and the release of corticotropin releasing hormone (CRH), adrenocorticotropin releasing hormone (ACTH), and cortisol (CORT), but it also can be defined as any psychological alteration from homeostasis (Burchfield, 1979). A stressor is any environmental event that induces a release of these stress hormones or causes the psychological perturbation. Such stressors

can include loss of a marriage or a loved one, legal stress, demanding or hazardous work environments, family stress, and low income (Stephens & Wand, 2012; Thoits, 2010). Anxiety, in contrast to stress, is a feeling or perception of unease, which is manifested in behavioral alterations but may not always be accompanied by elevated levels of stress hormones.

Activation of the hypothalamic-pituitary-adrenal (HPA) axis defines the body's chemical response to stress. The three structures involved in this axis are the paraventricular nucleus (PVN) of the hypothalamus, corticotrophic cells of the anterior pituitary gland, and the adrenal glands. Activation of the stress response, including basal levels of PVN CRH and glucocorticoid release, is higher in females than in males (Critchlow, Liebelt, Bar-Sela, Mountcastle, & Lipscomb, 1963; Handa, Burgess, Kerr, & O'Keefe, 1994; Iwasaki-Sekino, Mano-Otagiri, Ohata, Yamauchi, & Shibasaki, 2009; Kitay, 1963; Rhodes, Kennell, Belz, Czambel, & Rubin, 2004; Viau & Meaney, 1991). The hypothalamus releases CRH through the hypopheseal portal system to the anterior pituitary gland. In response to environmental perturbations, CRH release stimulates the transcription of the proopiomelanocortin gene, which produces precursors for both ACTH and β-endorphin. ACTH acts at the adrenal cortex to stimulate the release of glucocorticoids, primarily cortisol in humans and corticosterone in rodents (both referred to as CORT). CORT goes on to mobilize energy resources necessary to cope with the environmental perturbation, and also returns to the brain where it inhibits the PVN from releasing additional CRH through negative feedback. Neither CRH nor CORT are

restricted to only acting in the HPA axis, but for the purpose of this discussion, activity only in several key stress-related brain regions will be considered.

The extended elevation of CRH from chronic stress elicits desensitization in males through the internalization of CRH-1 receptors (CRH-R1) in the locus coeruleus (LC), a brain region necessary for alcohol-induced elevation of ACTH (Bangasser et al., 2010; Selvage, 2012). Subsequent stressors then must be stronger in order to be recognized by the body, but higher levels of stress hormones take a longer time to clear after the stressor ends, increasing the exposure of the body to high levels of glucocorticoids and furthering the desensitization of receptors (Stephens & Wand, 2012). Females, on the other hand, upregulate and externalize CRH-R1 in response to stress, which may sensitize them to future stressors and cause elevation in anxiety behaviors (Bangasser et al., 2010; Bangasser, 2013). Despite this sex difference in CRH receptor mediation in response to stress, even in females, the HPA axis becomes dysregulated if stress is maintained and may result in decreased responsivity to stressors. The development of low cortisol responsivity, an insensitivity to cortisol even when high levels are present, is characteristic of the transition into alcohol dependence (Koob & Le Moal, 2001). This blunted reactivity of the stress response system strongly implicates the dysregulation from excessive stress as an important factor to consider in individuals with AUD (Walther, Rice, Kufert, & Ehlert, 2016).

Negative emotionality, including states of stress and anxiety, is often reported as a motivating factor behind alcohol and drug consumption (Richardson, Lee, O'Dell, Koob, & Rivier, 2008). Paradoxically, alcohol consumption induces cortisol release, suggesting

that alcohol itself activates the stress response and is therefore a stressor. Many other drugs of abuse also activate the stress response, potentially implicating activation of the stress response in the reinforcing effects of drug use. This is also supported by the observation that glucocorticoids alone also have rewarding effects and may even be a necessary factor in the release of dopamine during rewarding activity (Piazza & Le Moal, 1997; Saal, Dong, Bonci, & Malenka, 2003). Removing the source of CORT through adrenalectomy inhibits high alcohol self-administration, but administering exogenous CORT restores high levels of alcohol administration, suggesting that CORT is necessary for high alcohol self-administration (Deroche, Marinelli, Le Moal, & Piazza, 1997).

The ability for alcohol to alleviate anxiety is potentiated by stressful experience, and does not significantly alter anxiety-related behaviors in animals who did not experience a stressor (Tran, Nowicki, Fulcher, Chatterjee, & Gerlai, 2016). Self-medication of stress may motivate alcohol consumption, but stress independently increases the rewarding properties of ethanol, as demonstrated by increased conditioned place preference acquisition (Bahi, 2013; Stephens & Wand, 2012). The relationship between alcohol and stress is clearly paradoxical and complex in many ways, but from here alcohol will primarily discussed in relation to its stress-alleviating properties.

IV. Gonadal Hormones

As a parallel to the HPA axis controlling the stress-hormones, the hypothalamo-pituitary-gonadal (HPG) axis controls the production of gonadal hormones. The

hypothalamus releases gonadotropin releasing hormone (GnRH) to the anterior pituitary, which releases gonadotropic hormones; either lutenizing hormone (LH) or follicle stimulating hormone (FSH). These two tropic hormones stimulate the gonads in males and females to release androgens or estrogens, stimulation that is largely dependent on the pulsatile patterns of LH and FSH release from the pituitary. In females, androgens are first produced by the theca cells of the ovary before the granulosa cells convert them to estrogens that are then circulated throughout the body. Most of the negative feedback that occurs in the brain for the HPG axis is mediated through estrogen receptors (ERs), rather than androgen receptors.

In the discussion of hormones involved in sex differences in stress-related consumption, testosterone, dihydrotestosterone (DHT), and estrogens are particularly relevant. Testosterone and DHT are androgens, released primarily from the testes in males, while estrogens are the released primarily from the ovaries in females. Each of these hormones can also be synthesized de novo in the brain and can be locally converted from circulating steroid hormone precursors. Testosterone, for example, is converted to the estradiol, the primary estrogen, through an enzyme called aromatase. This conversion through aromatase occurs in many places in the brain to provide feedback from testosterone.

Gonadal hormones have both activational and organizational roles in the brain and body. Organizational effects are permanent effects on structure and function. Gonadal hormones begin establishing their organizational effects a matter of weeks after conception. Traditional knock out models allow for organizational effects of a hormone

to be studied by removing a gene and constitutively eliminating its presence. Activational effects, in contrast, are dependent upon the hormone at a given point in time. Secondary sex characteristics that set in with the escalation of gonadal hormones and disappear with the removal of gonadal hormones are examples of activational sex hormone effects. Activational effects can also be studied through the use of receptor agonists and antagonists after development, or in the case of gonadal hormones, can be studied through castration and ovariectomy. Castrating a male rodent elevates stress responsivity and basal CORT and ACTH activity. Replacement with exogenous testosterone can reverse this female-pattern stress responsivity and restores male-like behavior, revealing that testosterone has an activational role in producing male-typical stress responsivity (Handa et al, 1994b; Viau & Meaney, 2004).

The relatively higher risk for women to develop psychiatric disorders like anxiety or AUD may either be attributable to ovarian hormones increasing risk in women, or testicular hormones reducing risk in men. Low circulating sex steroids combined with elevated glucocorticoids increase the risk of men developing psychopathology, such as depression (Walther et al., 2016). Androgens in isolation generally are anxiolytic (Almeida, Waterreus, Spry, Flicker, & Martins, 2004; Amore, 2004; Rizk, Robertson, & Raber, 2005). Chronic alcohol administration reduces overall testosterone levels in male rats, perhaps removing the protective factor against high ethanol consumption (Rachamin et al., 1979). This potentially explains the observation that males who are chronically exposed to alcohol exhibit female patterns of alcohol metabolism. Prior to reaching sexual maturity at puberty, both sexes exhibit the same level of alcohol dehydrogenase

activity and alcohol metabolism. Alcohol dehydrogenase is the enzyme responsible for breaking down alcohol in the blood. However, following puberty at 10 weeks in rats, when levels of gonadal hormones rise drastically, male alcohol dehydrogenase activity and metabolism slows compared to females (Rachamin et al., 1979). Removing male testes prior to puberty prevented the drop off in male metabolism rate; removing ovaries did not change female metabolism rate, suggesting that the presence of testosterone leads to decreased metabolism in males, rather than the presence of estrogen leading to increased metabolism in females (Rachamin et al., 1979). Chronic exposure to ethanol maintained elevated alcohol dehydrogenase activity in males, indicating that perhaps in chronic exposure, the sex difference in alcohol metabolism rates is eliminated. This result may also be elicited by the fact that chronic ethanol exposure causes a decrease in testosterone production (Maneeshm Dutta, Chakrabarti, & Vasudevan, 2006; Mendelson, Mello, & Ellingboe, 1977)

As with all other hormones, the diversity of specific physiological responses to gonadal hormones is mediated by varied types of receptors for a specific ligand. Receptor variety allows for a single hormone to elicit multiple effects depending on which receptor it interacts with. The PVN has both androgen and estrogen receptors, furthering the possibility that testosterone and estrogen mediate PVN activity directly (Handa & Weiser, 2014). Local conversion of testosterone to estrogen complicates the research on testosterone's importance by itself. DHT is therefore particularly useful in gonadal hormone research specifically because it is a non-aromatizable androgen and binds to the same receptors as testosterone. In studies that show administration of testosterone

reduces stress sensitivity, non-aromatizable DHT administration showed the same results, demonstrating that testosterone's role in protecting against stress-reactivity is not dependent upon local aromatization to estradiol (Lund, Munson, Haldy, & Handa, 2004a; Viau, 2002). Additionally, blocking activity of 5a-reductase, the enzyme that converts testosterone to DHT, increases stress reactivity in a manner similar to castration, suggesting that testosterone's protective roles in the stress system may actually be dependent upon local conversion to DHT (Handa, Kudwa, Donner, McGivern, & Brown, 2013).

Until Kuiper and colleagues in 1997 discovered ERβ, there was only hypothesized to be one estrogen receptor, ERα, which would have limited the potential for estrogen to elicit varied responses (Kuiper et al., 1997). The action of ligands at ERα and ERβ oppose each other in many cases, including regulation of the stress response (Handa & Weiser, 2014). The difference between the two receptors then largely comes down to where each is expressed. Along with high expression in female reproductive organs, ERα is present in moderate to high levels in the pituitary and the adrenal (Kuiper, Shughrue, Merchenthaler, & Gustafsson, 1998). ERα activation enhances the release of CORT and inhibits the activity of gamma-aminobutyric acid (GABA) neurons that normally maintain a negative tone on the HPA axis. Loss of negative tone on the axis could be responsible for the increased stress sensitivity of females (Weiser & Handa, 2009).

ERβ, on the other hand, is expressed in moderate to high levels in the lung, bladder, and brain (Kuiper et al., 1998). ERβ is expressed in the PVN, where binding mitigates CRH release (Lund, Hinds, & Handa, 2006). Ovariectomized females treated

with diarylproprionitrile, an ERβ selective agonist, demonstrated decreased CORT and ACTH response to stressors (Lund, Rovis, Chung, & Handa, 2005). Although the β receptor is classified as an estrogen receptor, current research postulates that a steroid metabolite, 3b-diol, rather than estrogen may be the endogenous ligand for the receptor (Weihua, Lathe, Warner, & Gustafsson, 2006). Anxiolytic effects of androgens could be explained through androgen's ability to inhibit the HPA axis through either androgen or ERβ receptors. Androgen action at ERs is not significantly caused by aromatization to estrogen, but rather it can act through local conversion to DHT. 3b-diol is a primary product of DHT production that has low affinity for androgen receptors, but strong affinity for ERβ (Kuiper et al., 1997). The enzyme for reducing DHT to 3b-diol is present in the PVN, so any DHT that reaches the PVN would be converted to this ERβ-reactive metabolite (Lund et al., 2006). Direct administration of 3b-diol without DHT is sufficient to reduce CORT and ACTH levels (Lund, Munson, Haldy, & Handa, 2004b). This effect could not be replicated in ERβ null mice, which indicates the effect is directly mediated by 3b-diol action at ERβ (Oyola et al, 2011).

The differences in ERα and β expression and activity pose a potential mechanism for the sex-difference in stress responsivity. Estrogen in females acts at high density ERα receptors in the pituitary and adrenals to elicit high levels of ACTH and CORT. Testosterone cannot act at these ERα receptors, but can act at ERβ receptors through the conversion to DHT and 3b-diol. Binding at ERβ receptors in the PVN causes a decrease in HPA activity. The net result is an ERα-mediated increase in stress reactivity in females, and an ERβ-mediated decrease in stress reactivity in males.

V. β-Endorphin

β-endorphin, an endogenous opioid peptide, modulates the hypothalamic-pituitary-adrenal (HPA) axis (Charmandari, Tsigos, & Chrousos, 2005; Pechnick, 1993; Sarkar, Kuhn, Marano, Chen, & Boyadjieva, 2007) by inhibiting secretion of CRH (Buckingham, 1986; Plotsky, 1991; Sarkar et al., 2007). β-endorphin also contributes to behavioral stress responses in a number of ways, including promotion of cataleptic behavior in forced-swimming and stress-induced analgesia (Amir, 1982; Ribeiro, Kennedy, Smith, Stohler, & Zubieta, 2005; Yamada & Nabeshima, 1995). Our lab has shown that mice lacking this peptide exhibit exaggerated behavioral responses to stressors (Barfield, et al., 2010; Barfield, Moser, Hand, & Grisel, 2013; Grisel, et al., 1999). Moreover, a clinical correlation has been established between heritable levels of β-endorphin and risk for excessive drinking (Froehlich, Harts, Lumeng, & Li, 1990; Gianoulakis, 2009; Wand, Mangold, El Deiry, McCaul, & Hoover, 1998). This body of research supports the contention that β-endorphin contributes to the relationship between stress and alcohol.

Several brain regions beyond the PVN are implicated in the relationship between stress and alcohol as it pertains to β-endorphin. The ventral hippocampus (VH) has been implicated in anxiety-like behavior, with mice completely lacking β-endorphin having the most anxious phenotype and highest levels of CRH messenger ribonucleic acid (mRNA) (Barfield, et al., 2010; Grisel, Bartels, Allen, & Turgeon, 2008). Such data suggest

chronic upregulation of the HPA axis associated with β-endorphin deficiency (Rubinstein, et al., 1996). The ventral hippocampus sends glutamatergic projections to the basolateral amygdala, linking the excitation of these two regions (Stamatakis, et al., 2014). The amygdala and bed nucleus of the stria terminalis (BNST) also play a major role in assessing stressors and helping to coordinate anxiety-related behaviors. The amygdala is activated by stress and has glutamatergic projections that lead to both the medial prefrontal cortex and the BNST. The BNST, located between the amygdala and the nucleus accumbens, connects stress and reward centers of the brain. Hyperexcitability of the BNST has been seen in chronic drug use and is associated with increased stress susceptibility (Silberman & Winder, 2013; Stamatakis, et al., 2014). The BNST sends CRH to the paraventricular nucleus of the hypothalamus, which is an excitatory signal increasing release of ACTH and CORT (Myers, et al., 2015). The dorsomedial prefrontal cortex (dmPFC) contributes to processing of emotionally salient information and glutamatergic projections extend from here to the amygdala and BNST.

VI. Exercise

Exercise, in a manner similar to environmental stress, impacts the body in a diverse number of ways. Exercise shares many reinforcing properties with addictive drugs and perhaps lowers alcohol preference. Endorphins are suggested to create the "runner's high" and are also implicated in the propensity to develop addiction. Recently, a phenomenon coined exercise dependence has emerged where the use of exercise has

come to closely resemble substance abuse (Leuenberger, 2006). Exercise tends to exacerbate alcohol-induced testosterone depletion in men, but there is not an interaction between cortisol reactivity to alcohol and exercise (Heikkonen et al., 1996). Mice with access to voluntary running display decreased alcohol consumption compared to sedentary mice (Ehringer, Hoft, & Zunhammer, 2009). However, alcohol remains a frequently abused drug in populations who exercise vigorously and regularly, such as athletes. Exercise, despite possibly encouraging elevated levels of consumption, may be capable of reducing some of the consequences of high alcohol consumption, such as oxidative damage (El-Sayed, Ali, & Ali, 2005).

VII. Summary of Purpose and Approach

Animal models have long been used to study the complex relationship between stress and alcohol, particularly as they relate to the dependent state (Becker, Lopez & Doremus-Fitzwater, 2011; Crabbe, 2014; Crabbe, Phillips & Belknap, 2010). Of course, animal models are not capable of modeling all aspects of human psychiatric disorders, but many aspects of human consumption are indeed well modeled in animals (Crabbe, Belknap, & Buck, 1994; Lester & Freed, 1973; Spanagel & Hölter, 1999; Cudd, 2005). For instance, the same factors that frequently cause recovering addicts to relapse – stress, small doses of the drug, and drug cues – can all induce relapse in rodent models of consumption (Epstein, Preston, Stewart, & Shaham, 2006; Shaham, Shalev, Lu, de Wit, & Stewart, 2003). Animals also provide a platform wherein more invasive studies can be

performed, which are necessary to identify molecular targets for pharmacological intervention in a given disorder (Spanagel & Holter, 2000; Bell et al., 2012).

The experiments in this study reported investigate the roles of ovarian and testicular hormones in producing the sex differences seen in vulnerability to alcohol consumption induced by exercise restriction. Voluntary exercise, most commonly modeled by free access to a running wheel in rodent studies, is a highly appetitive stimulus for rodents. Our model of exercise restriction likely induces an aversive state by removing a stimulus as appetitive as the running wheel. This model may generalize to other states of stressful experience, but there have not yet been studies conclusively defining exercise restriction as a stressor in rodent models. There are key similarities between exercise restriction and other methods of imposing stress in the lab that suggest the relevance of the exercise-restriction model to a source of stress. Exercise restriction is one of the limited environmental conditions that are capable of eliciting an increase in ethanol consumption, similar to stress-induced increases in alcohol consumption that are seen in the clinic. The sex difference seen in other models of stress where females are more likely to administer alcohol in response to stress than males (Agaibo et al., 2016; Rivier, 1993) is also seen in exercise restriction (Piza-Palma et al., 2014).

We hypothesize that both testicular and ovarian hormones are involved in the regulation of this sex difference, wherein females are more likely to increase alcohol consumption in response to a locked running wheel (Piza-Palma et al., 2014). We hypothesize that ovarian hormones promote the high levels of exercise restriction-induced alcohol consumption seen in females and removing ovarian hormones through

ovariectomy will decrease this tendency to self-medication aversion from wheel restriction with alcohol. Similarly, we hypothesize that testicular hormones play a protective role against exercise restriction-induced alcohol consumption and therefore removing the testes should enhance male vulnerability to drink alcohol when restricted from accessing their running wheel.

We also investigated the role of β-endorphin as a potential risk factor for female engagement in exercise restriction-induced alcohol consumption, as well as the interaction between β-endorphin and testicular hormones in males. We hypothesize that β-endorphin is central to the ability to self-medicate aversive effects of wheel restriction and that β-endorphin deficiency will increase general experience of stress but could also limit the efficacy of increased alcohol consumption in reducing such aversion. Effective self-medication is reflected by high levels of alcohol consumption causing an increase in anxiety behaviors or stress reactivity. We expect limited efficacy of self-medication to be revealed through a lack of increased consumption in response to exercise restriction. The effects of β-endorphin may be contingent upon the presence of testosterone in males, with loss of one or the other endocrine factor altering susceptibility to exercise restriction-induced alcohol consumption. The experiment exploring the relationship between β-endorphin and testosterone is largely exploratory in nature and in response to previous unpublished findings in our laboratory, suggesting testosterone-dependent effects of β-endorphin in alcohol sensitivity.

General Methods

Subjects

Male and female C57BL/6J (B6) mice were used for all experiments presented. All were adults over 7 weeks old, the average age of reproductive maturity, when experimental conditions began. When gonadectomy surgery occurred, animals were a minimum of 49 days old. This ensured that our manipulations were exclusively examining the activational effects of gonadal hormones. Mice were generated in-house from stock obtained from the Jackson Laboratories (Bar Harbor, ME). Mice were weaned at 21 days and group housed by sex in Plexiglas cages filled with corncob bedding in a 12 hour reverse light:dark cycle (lights off at 0930 for the majority of experiments, but the 12 hour cycle was altered for researcher's schedule within each semester). Colony and experimental rooms were maintained at 21±2°C. The housing conditions were the same during the 14-day experimental period except that subjects were moved to single housing with standard mouse chow and tap water, both available at all times. The bedding was changed once during the experiment, between the 4-day baseline and 10-day experimental periods. Each cage was also equipped with a running wheel, which measured rotations in one-minute bins.

Protocol

All protocols were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee at Bucknell University.

Ovariectomy surgical procedure

The ovariectomy procedure used was detailed in by Grisel and colleagues (Grisel, Allen, Nemmani, Fee, & Carliss, 2005). Sham and ovariectomized (OVX) mice were anesthetized with a mixture of 100mg/kg ketamine and 12mg/kg xylazine, administered intraperitoneally. Dorsolateral incisions were made through the skin and muscle to expose ovaries. In ovariectomized mice, ovaries were ligated and excised. Muscle was sutured with 6-0 chromic gut suture (Covidien, Dublin) and skin was sutured with 3-0 silk suture (Covidien, Dublin). Baytril (enrofloacin: antibiotic, administered 5 mg/kg) and Rimadyl (carprofen: anti-inflammatory, administered 5mg/kg) were administered sub-cutaneously following surgery. Sham mice had dorsolateral incisions made and ovaries exposed, but remained intact. Sham animals additionally received Baytril and Rimadyl. Naïve females received saline injections at the time of surgeries and were single-housed in the same protocol as the post-operative mice. Mice were single-housed for 24 hours after surgery to allow for incisions to heal, then mice were regrouped to limit anxiety induced by individual housing in females during the remaining recovery time (Palanza, Gioiosa, & Parmigiani, 2001; Detillion, Craft, Glasper, Prendergast, & DeVries, 2004). Two weeks elapsed between surgery and the beginning of the experimental procedure to allow for hormone depletion, a time period demonstrated to be sufficient to deplete gonadal hormones below levels detectable in the plasma (Wichmann, Zellweger, DeMaso, Ayala, & Chaudry, 1996).

Castration surgical procedure

Sham and Castrated (CAST) mice were anesthetized with a mixture of 100mg/kg ketamine and 12mg/kg xylazine, administered intraperitoneally. A ventral incision was made through the scrotal skin to access the testes. Each testis was exteriorized and excised from the scrotum before the incision was sutured. Sham mice did not have testes excised. Skin was sutured with 3-0 silk suture (Covidien, Dublin). Baytril (enrofloxacin: antibiotic, delivered 5mg/kg) and Rimadyl (carprofen: anti-inflammatory, delivered 5mg/kg) were administered sub-cutaneously following surgery. Naïve males received saline injections at the time of surgeries and were single housed in the same protocol as the post-operative mice. Mice were single-housed for 24 hours after surgery to allow for incisions to heal, then mice were regrouped with cage mates during the remaining recovery time (Palanza et al., 2001; Detillion et al., 2004). Two weeks elapsed between surgery and the beginning of the experimental procedure to allow for hormone depletion, a time period demonstrated to be sufficient to deplete gonadal hormones below levels detectable in the plasma (Wichmann et al., 1996).

β-endorphin deficient model

The β-endorphin-deficient model was developed about 20 years ago in the laboratory of Malcolm Low (Rubinstein et al., 1996) by insertion of a premature stop codon into the Pomc gene. The gene mutation has been fully backrossed to the B6 strain (>20 generations). Homozygous knockouts (βE-KO) cannot synthesize β-endorphin (βE), though all other products of the POMC protein show normal expression. Opioid receptor

expression also remains unchanged (Rubinstein et al., 1996). The model has been used in studies of metabolism, as βE-KO males show an altered growth curve resulting in increased body mass and white fat (Low, Hayward, Appleyard, & Rubinstein, 2003). We previously suggested hypersensitivity to stress in βE-deficient mice (e.g., Barfield, et al., 2013) but no overt alterations in the HPA axis have been reported, and homozygous mutant mice appear otherwise normal in terms of development and behavior. Heterozygous (βE-HT) mice for the βE knock out gene produce 50% of B6 levels of βE.

Drinking and wheel procedure

A modified drinking in the dark procedure (Ehringer, et al., 2009; Rhodes, et al., 2007) was used in this study. Mice were allowed access to 20% EtOH (v:v in tap water) for two hours each day, beginning three hours into their dark cycle. EtOH was presented in a two-bottle free access paradigm with no water deprivation. EtOH presentation was switched every two days to prevent the development of side preference. The first four days of EtOH self-administration were considered baseline days, during which the animals habituated to their cages and acquired self-administration behavior. Data for baseline is shown, but was not analyzed because it represented this acquisition period. This baseline period was followed by 10 days of experimental trial where wheel accessibility was manipulated to examine the effect of exercise restriction on EtOH consumption. The running wheel was available to the mice for voluntary exercise with the exception of every other day when the wheel was locked for all mice. On days of exercise restriction, the wheel was locked for a total of three hours, beginning one hour before, and continuing throughout the EtOH access period.

Blood ethanol content (BEC) analysis

Following the final experimental manipulation, which was a locked wheel session, animals were lightly anesthetized for approximately 30s with isoflurane and sacrificed via rapid decapitation. Trunk blood was collected into Ethylenediaminetetraacetic acid (EDTA)-treated vacutainer tubes (Becton Dickinson, Rutherford, NJ). Blood was collected retro-orbitally for several runs of animals in experiments 1A and 1B before methodology was altered to allow for brain collection. Blood was centrifuged at 3000 rpm for 30 min. at 4°C, and was then analyzed for BEC using Analox BEC Analyzer (Analox Instruments Ltd., London, UK). The test uses alcohol oxidase enzyme, which oxidizes ethanol in the presence of molecular oxygen. The rate of oxygen consumption is directly proportional to the alcohol concentration. Sensitivity of the analyzer is 0.1mg/dL.

Data and statistical analysis

Dosage of ethanol administration is reported in g/kg, representing the amount of solution consumed normalized for body mass. Body mass was measured at three points through the study to allow for more accurate calculation of g/kg.

$$g/_{kg} = \frac{(mls\ EtOH\ solution\ consumed)(EtOH\ density)(EtOH\ concentration)}{body\ mass\ in\ kg}$$

Preference is the percentage of total fluid intake from the bottle with the ethanol solution

$$preference = \frac{(mls\ EtOH\ solution\ consumed)}{(mls\ total\ fluid\ consumed)} * 100$$

Average consumption was calculated by averaging g/kg or preference across the entire ten-day experimental period, regardless of wheel availability. Change scores for g/kg and preference were calculated by subtracting the average consumption on unlocked days from the average consumption on locked days.

Behavioral data, and BECs were analyzed with SPSS (IBM 2013, version 23.0) using condition – either genotype or gonadal condition, first in a repeated measure analysis of variance (ANOVA) to evaluate changes across the ten-day experimental period. Hypotheses that a locked wheel would increase alcohol consumption compared to locked days was tested within each genotype through one-sample, one-tailed t-tests. One-way ANOVA was used to measure variation between total, or one-time measurements for each condition, such as average EtOH consumption. Specific between-group comparisons were performed using Tukey honest significant difference (HSD) post-hoc test where appropriate. Correlations were evaluated using Pearson's Correlation. In all cases the α-level was set at 0.05.

Experiment 1A Introduction

This experiment was intended to investigate the role of ovarian hormones in the tendency for females to self-administer relatively higher quantities of alcohol in response to exercise restriction. Manipulation of ovarian hormones through ovariectomy was used to examine this role in a model of drinking that utilized exercise restriction as an imposed aversive stimulus. We hypothesized that ovarian hormones contribute to the escalated vulnerability to elevated alcohol consumption in response to exercise restriction and that ovariectomized females would demonstrate a lower vulnerability, as evidenced by a decreased tendency to increase consumption on days of exercise restriction.

Experiment 1A Methods

Subjects

Fourty-seven female C57BL/6J (B6) mice, average age 152 days ± 14 days were used in this experiment. Subjects included 14 naïve, 16 sham, and 17 ovariectomized (OVX) mice. Housing conditions were consistent before and during the experiment, except that subjects were moved to single housing in TSE Phenomaster Plexiglas cages that contained a food hopper filled with standard mouse chow as well as a tube of tap water, both available at all times. The cages were also equipped with a running wheel 11cm in diameter (TSE Systems, Bad Homburg, Germany).

Surgical Procedure

Mice over the age of 58 days underwent ovariectomy surgical procedures detailed in the general methods section. The average age of mice at the time of surgery was 139 days ± 14 days.

Drinking and wheel procedure

The wheels were locked via a mechanical braking device that is controlled by the TSE PhenoMaster program. The alternating days of differential wheel access are referred to as locked and unlocked days. The TSE PhenoMaster program was intended as our method for collecting both drinking data and running data, but the program generated unreliable and incorrect consumption data. Therefore, the TSE PhenoMaster program measured only running data and drinking was assessed manually, by reading gradations on a 13mL tube with a ball bearing sipper. Each day we calculated the dosage administered by each mouse as well as EtOH preference during the 2hrs of EtOH access. Twelve mice could be tested at a time in our facility, so the total testing was run in four trials over the course of one year. Condition (OVX, sham, naïve) was distributed as equally as possible across experimental trials, but the numbers varied slightly in each run to ensure that post-op mice could be regrouped with siblings of the same condition.

Experiment 1A Results

Daily alcohol dosage and preference were variable over the course of the experiment (Figs. 1A & 1B). Repeated-measure ANOVA of daily drinking behavior through the ten-day experimental protocol revealed a main effect of time on g/kg ($F[9,369] = 11.101$, $p < 0.001$) and preference ($F[9,360] = 2.584$, $p < 0.01$), both tending to increase over the course of the experiment. There was also a main effect of gonadal condition in daily g/kg consumption, driven by ovariectomized mice drinking significantly less than naïve mice ($F[2,41] = 4.361$, $p < 0.05$), but changes in consumption did not depend upon condition across the experimental period ($F[18,369] = 0.363$, $p > 0.05$). The effect of condition was not present in preference ($F[2,40] = 1.081$, $p > 0.05$) and there was no interaction between time and condition in daily preference ($F[18,360] = 0.770$, $p > 0.05$).

Because there were no unique effects of ovariectomy on drinking over time, all 10 experimental days were collapsed to evaluate the effect of ovariectomy on average alcohol dose and preference (Figs. 2A & 2B). One-way ANOVA showed that gonadal condition significantly altered total g/kg alcohol consumption ($F[2,44] = 3.031$, $p = 0.05$) with *post-hoc* Tukey HSD revealing that ovariectomy significantly decreased consumption from naïve ($p < 0.05$). A parallel trend can be noted in total preference, although the genotypes did not significantly differ from each other ($F[2,44] = 1.122$, $p > 0.05$).

Change scores were our primary measure of susceptibility to increased alcohol consumption in response to exercise restriction and were calculated by subtracting averaged consumption on unlocked days from averaged consumption on locked days (Figs. 2C & 2D). All three conditions displayed susceptibility to exercise restriction-induced alcohol consumption, as evidenced by one-sample *t*-tests comparing the change score to the null hypothesis of zero, which would indicate no change in response to exercise restriction (naïve mice: $t[13] = 6.318$, $p < 0.001$; sham mice: $t[15] = 3.647$, $p < 0.005$; ovariectomized mice: $t[16] = 3.931$, $p = 0.001$). One-way ANOVA analysis showed that this tendency to increase consumption in response to exercise restriction did not differ by condition ($F[2,44] = 0.366$, $p > 0.05$). Changes in preference mirrored those observed in g/kg in most cases, significantly increasing in response to exercise restriction for naïve and sham animals, but not for ovariectomized mice (naïve mice: $t[13] = 3.418$, $p = 0.005$; sham mice: $t[15] = 2.196$, $p < 0.05$; ovariectomized mice: $t[16] = 1.327$, $p > 0.05$). In this measure as well, one-way ANOVA did not reveal any difference between conditions on the degree to which they increased consumption ($F[2,44] = 0.365$, $p > 0.05$).

Average running was impacted by ovariectomy in these animals (Fig. 3; $F[2,21] = 6.112$, $p < 0.01$), although sham surgery may have had a slight inhibitory effect on running behavior by itself. *Post-hoc* Tukey HSD revealed a significant difference between ovariectomy and naïve groups ($p < 0.01$), although the intermediate sham group did not significantly differ from either ovariectomy or naïve animals. No differences in overall alcohol consumption or exercise restriction-induced alcohol consumption were

explained by the differences in running behavior. Therefore, we do not believe that the differences in running reflect a difference in the salience of exercise as a stimulus.

One-way ANOVA comparing BECs between groups revealed no differences based on gonadal condition (Fig. 5A; $F[2,32] = 1.085$, $p > 0.05$). We did observe the expected relationship between alcohol consumed on the final day of the experience and BEC (Fig. 5B; Pearson correlation, $r = 0.470$, 2-tailed $p < 0.005$). Taken together, these indicate that ovariectomy does not produce differences in alcohol metabolism.

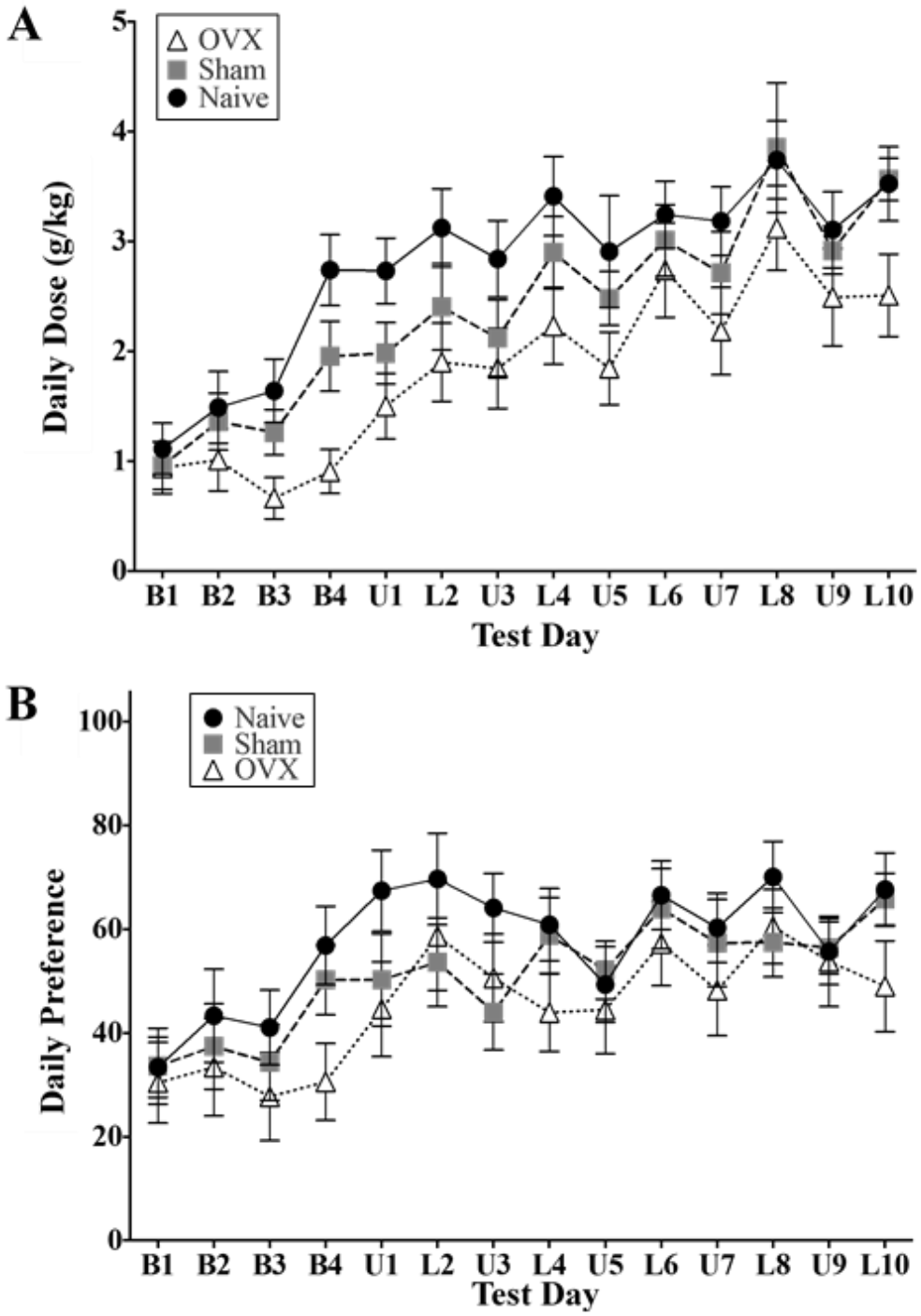

Figure 1. Group means ± standard error of the mean (SEM) for daily g/kg alcohol consumption (A) and preference (B). “B” signifies baseline days, “U” signifies unlocked days, “L” signifies locked days.

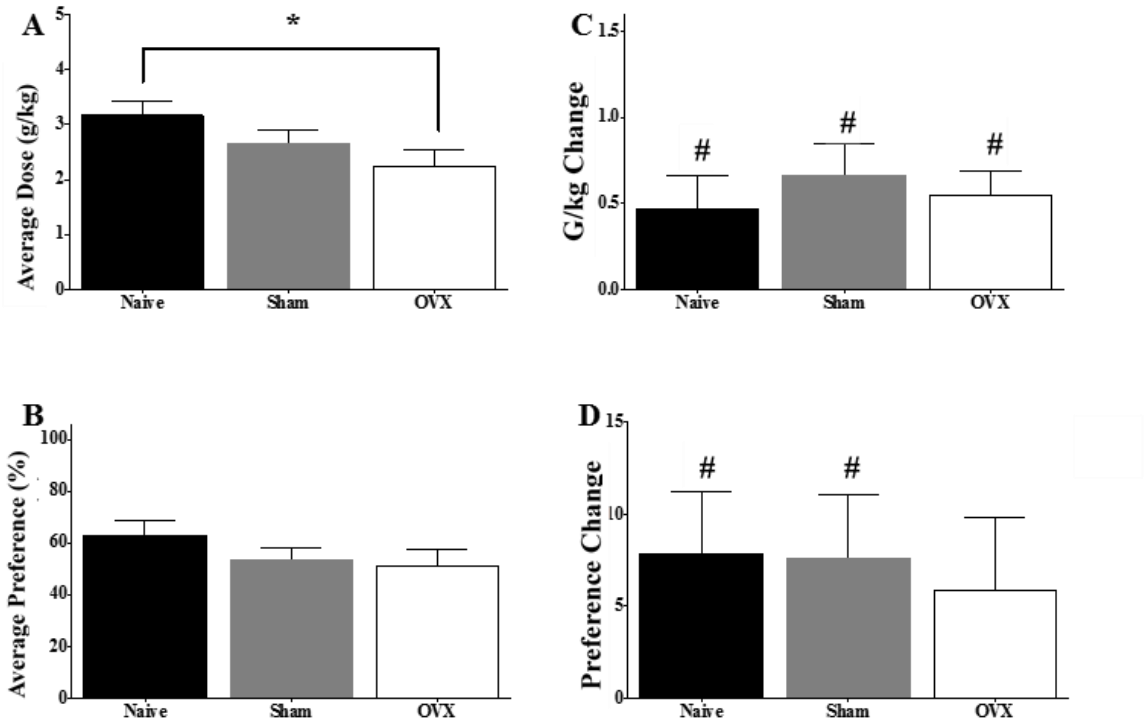

Figure 2. Group means ± SEM for average alcohol consumption (A; g/kg) and preference (B), collapsing across the entire 10-day experimental period. Change, in g/kg consumption (C) and preference (D), between averages of locked and unlocked days by gonadal condition. *denotes significance between naïve and ovx groups at $p < 0.05$, # denotes significant difference from zero at $p < 0.05$.

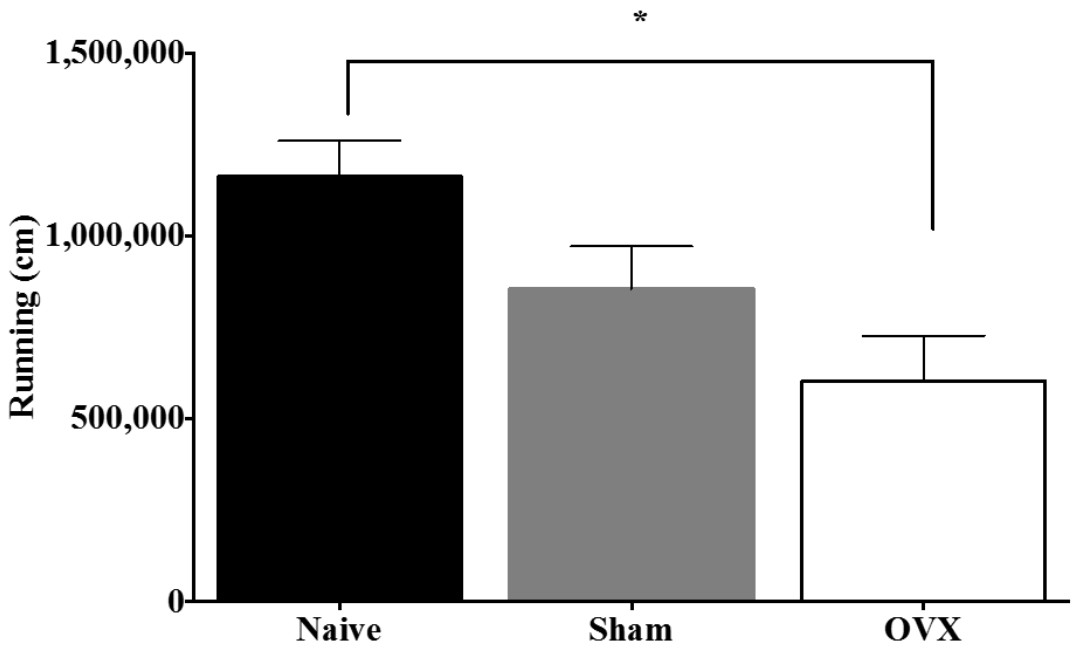

Figure 3. Group means ±SEM for daily running behavior, expressed in cm, collapsed across all days of the experiment. *denotes significance between naïve and ovx groups at $p < 0.05$.

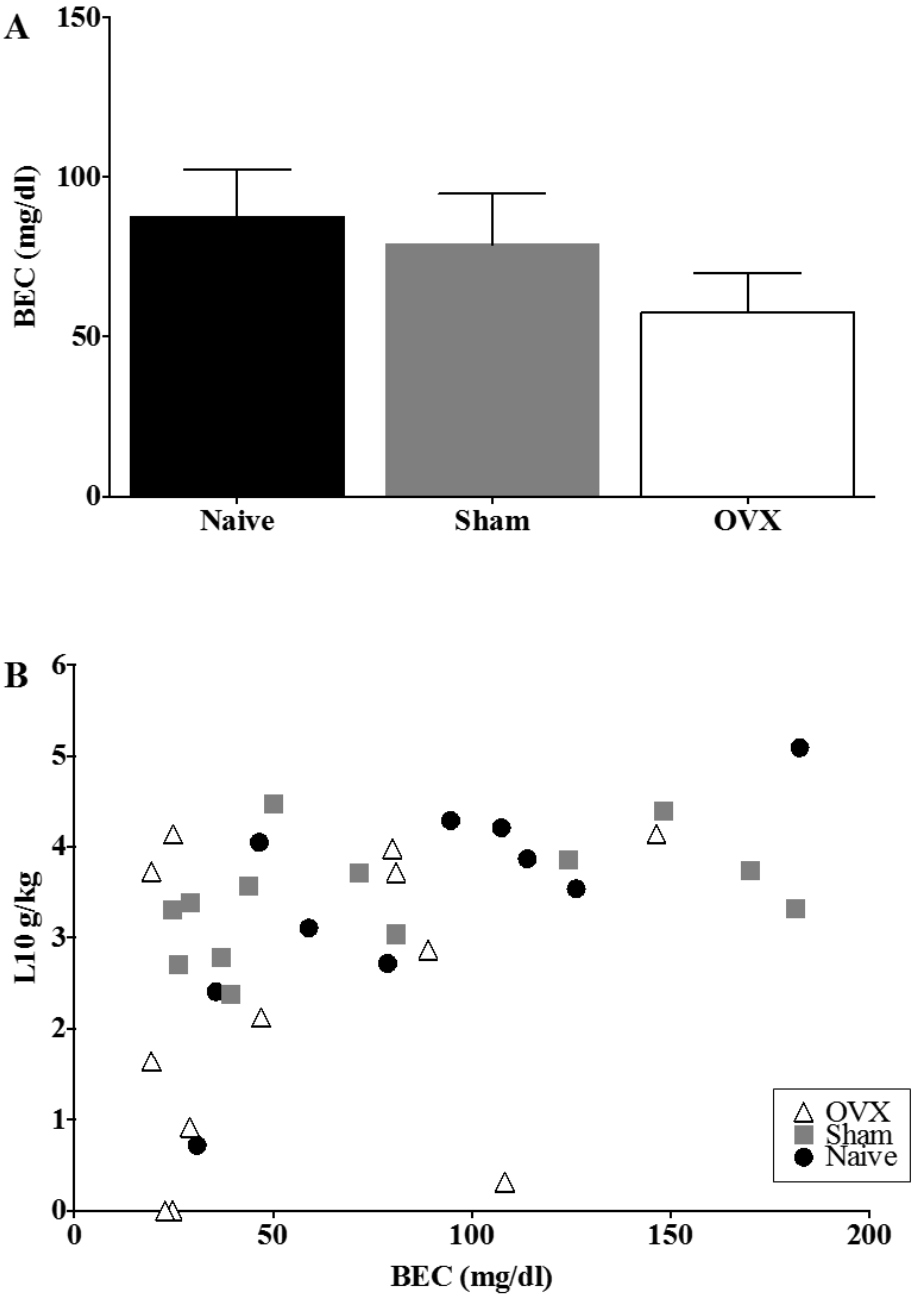

Figure 4. A shows the average (± SEM) blood ethanol content (BEC) in each gonadal condition immediately following the final day of EtOH exposure (L10). B shows the correlation between alcohol consumed on the final day and resultant BEC ($r = 0.470$, $p < 0.005$).

Experiment 1A Discussion

Despite our hypothesis that ovarian hormones were responsible for female-typical increases in alcohol consumption in response to exercise-restriction, animals in naïve, sham, and ovariectomized groups drank equivalently on free exercise and exercise-restricted days. Each group was equally susceptible to exercise-restricted alcohol consumption, significantly increasing alcohol consumption on days when our exercise-restriction was applied. We found that while ovarian hormones may play a mild role in the tendency for females to drink more alcohol on average, they did not impact exercise restriction-induced alcohol consumption.

Gonadectomy has been shown to result in depressed physical activity, an effect related to the absence of gonads and reversed with re-implantation of the gonadal tissue or injection of gonadal tissue extract (Richter, 1993). Despite depressed running behavior in ovariectomized mice, exercise-restricted changes in alcohol consumption remained equivalent to those of naïve and sham animals. Moreover, the running data averaged across condition did not correlate significantly with either average drinking or exercise restriction-induced changes in drinking, suggesting that the tendency to engage in voluntary exercise did not predict alcohol preferences.

Sham animals represented an intermediate phenotype that was not significantly different from either the naïve animals or the ovariectomized animals. Because the sham animals did not drink the same amount of alcohol as the naïve animals, it is difficult to conclude whether the difference between naïve and ovariectomy are due entirely to the

presence of ovarian hormones, or whether there is an effect of surgery that could account for the differences.

There is robust literature on the interaction between ovarian hormones and the activity of alcohol dehydrogenase, the enzyme responsible for metabolizing ethanol in the body (Rivier, 1993; Sutker, Goist, & King, 1987; Teschke & Wiese, 1982). Our data suggest that ovariectomy did not alter alcohol metabolism, because the association for average group BEC did not covary with the average alcohol consumption and the correlation between different conditions did not differ from one other. This is consistent with much of the existing literature (Mezey, Potter, & Tsitouras, 1981; Rachamin et al., 1980). While there have been no studies directly examining the effect of exercise on alcohol metabolism rates in females, studies in males suggest that alcohol metabolism is not increased by exercise (Barnes, Cooke, King, & Passmore, 1965). Taken together, these pieces of evidence suggest that our results were not related to the effects ovariectomy altering the degree of intoxication that the animals experienced.

Experiment 1B Introduction

This experiment was intended to investigate the role of testicular hormones in the tendency for males to not increase alcohol self-administration in response to exercise-restriction. Manipulation of testicular hormones through castration was used to examine this role in a model of drinking that utilized exercise restriction as a stressor. We hypothesized that testicular hormones protect against vulnerability to exercise restriction-induced alcohol consumption and that castrated males would therefore demonstrate an escalated vulnerability, as evidenced by a heightened tendency to increase consumption on days of exercise-restriction.

Experiment 1B Methods

Subjects

Thirty-three male C57BL/6J (B6) mice with an average age of 99 days ± 15 days at the beginning of the experiment were used in this experiment. Subjects included 7 naïve, 11 sham, and 15 Castrated (CAST) mice. Housing conditions were consistent before and during the experiment, except that subjects were moved to single housing in TSE Phenomaster Plexiglas cages that contained a food hopper filled with standard mouse chow as well as a tube of tap water, both available at all times. The cages were also equipped with a running wheel 11cm in diameter (TSE Systems, Bad Homburg, Germany).

Surgical procedure

Mice underwent castration surgery as detailed in the general methods after 50 days of age. The average age of mice at the time of surgery was 85 days ± 15 days.

Drinking and wheel procedure

Alcohol was provided and running wheels were locked following the same design described in the general methods. The wheels were locked via a mechanical braking device that is controlled by the TSE PhenoMaster program. The alternating days of differential wheel access are referred to as locked and unlocked days. The TSE PhenoMaster program was intended as our method for collecting both drinking data and running data, but the program generated unreliable and incorrect consumption data. Therefore, the TSE PhenoMaster program measured only running data and drinking was assessed manually, by reading gradations on a 13mL tube with a ball bearing sipper. Each day we calculated the dosage administered by each mouse as well as EtOH preference during the 2hrs of EtOH access. Twelve mice could be tested at a time in our facility, and the total testing was run in 5 trials. The distribution of conditions across each trial was kept as consistent as possible, but numbers varied slightly in order to enable regrouping siblings of the same condition after post-op recovery. The five trials were run over the course of two years.

Experiment 1B Results

Condition did not impact the patterns of alcohol consumption across the period of the study. Daily drinking increased over time, as shown in by repeated measure analyses of the 10-day experimental period g/kg consumption (Fig. 5A; $F[9,252] = 2.477$, $p = 0.01$). There was, however, no main effect of effect of gonadal condition ($F[2,28] = 2.172$, $p > 0.05$) or an interaction between time variability and condition ($F[18,252] = 0.708$, $p > 0.05$). Similarly, there was a main effect of time on daily preference, with a tendency to increase preference over time (Fig. 5B; $F[9,261] = 3.118$, $p = 0.001$). There was no main effect of condition on daily preference ($F[2,29] = 1.047$, $p > 0.05$), and the effect of time did not interact with condition ($F[18,261] = 0.887$, $p > 0.05$).

Because daily drinking did not vary by condition, overall drinking tendencies were examined by collapsing data across the entire experimental period of locked and unlocked days. There was no effect, shown in one-way ANOVA, of genotype on total g/kg alcohol consumption (Fig. 6A; $F[2,30] = 1.461$, $p > 0.05$), and also not on total alcohol preference (Fig. 6B; $F[2,30] = 1.838$, $p > 0.05$). The standard error of the mean for the naïve group of mice is relatively large, possibly accounting for the inability to observe an effect of gonadal condition on overall drinking. There is a tendency for castration to increase overall alcohol consumption, as measured by g/kg consumption, although the sham is an intermediate phenotype.

Susceptibility to exercise restriction-induced alcohol consumption was quantified primarily through a difference score, calculated by subtracting the average consumption

on unlocked days from the averaged consumption on locked days. One-sample *t*-tests showed that all groups significantly altered consumption in response to the imposition of a locked wheel. Naïve and castrated animals significantly increased g/kg consumption in response to exercise restriction and shams tended to do the same (Fig. 6C; Naïve: $t[6] = 2.582$, $p < 0.05$; Sham: $t[10] = 1.709$, $p = 0.055$; Castrated: $t[14] = 1.8$, $p < 0.05$). Only sham animals significantly increased preference in response to locked wheel aversion (Fig. 6D; $t[10] = 3.449$, $p < 0.01$). Naïve and castrated animals did not significantly alter preference (Naïve: $t[6] = 1.578$, $p > 0.05$; Castrated: $t[14] = 1.590$, $p > 0.05$). One-way ANOVA revealed no difference between conditions on the increase in g/kg alcohol consumption ($F[2,30] = 0.400$, $p > 0.05$). There was also no effect of condition on change in preference on locked days ($F[2,30] = 1.608$, $p > 0.05$). However, in both the g/kg measure and the preference measure, shams tended to have much greater increases in response to the exercise restriction stressor.

One-way ANOVA demonstrated that there was no effect of condition of BEC when collapsing all animals of the same condition together into one average (Fig. 7A; $F[2,19] = 0.399$, $p > 0.05$). The overall correlation for BEC was moderate and positive (Fig. 7B; Pearson correlation $r = 0.478$, $p < 0.05$).

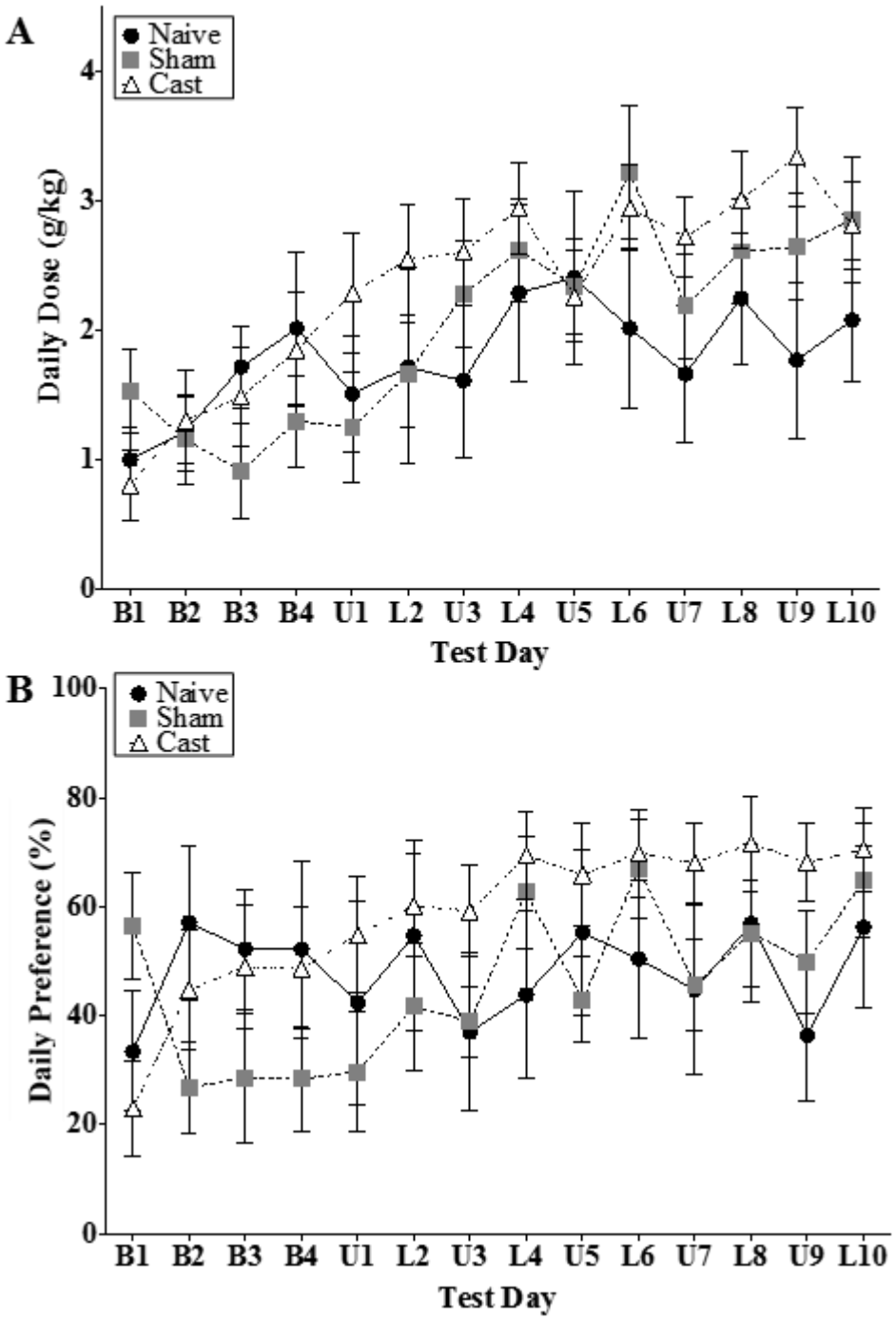

Figure 5. Group means ± SEM for daily g/kg alcohol consumption (A) and preference (B). "B" indicates baseline days, "U" indicates unlocked days, "L" indicates locked days.

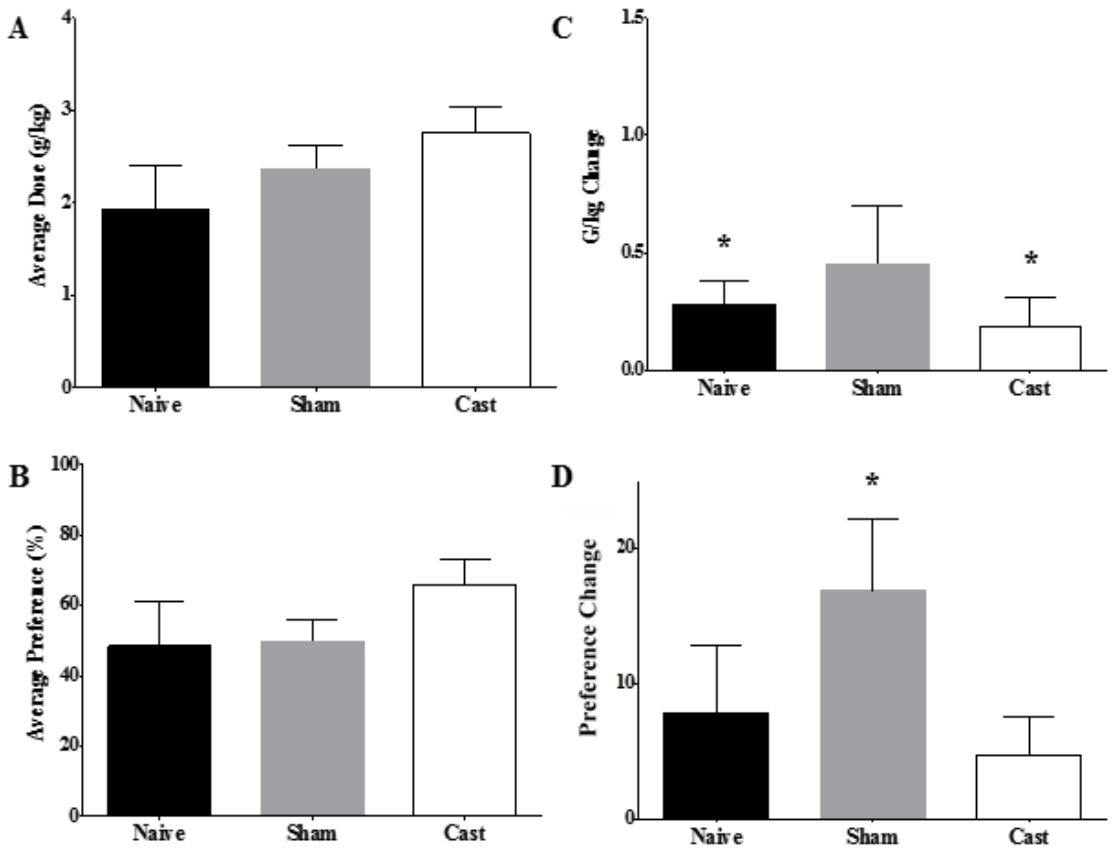

Figure 6. A & B show group means ± SEM for overall alcohol consumption (g/kg) and preference, collapsing across the entire 10-day experimental period. C & D show the difference, in g/kg consumption and preference, between averages of locked and unlocked days by gonadal condition. *denotes significance compared to zero and $p < 0.05$.

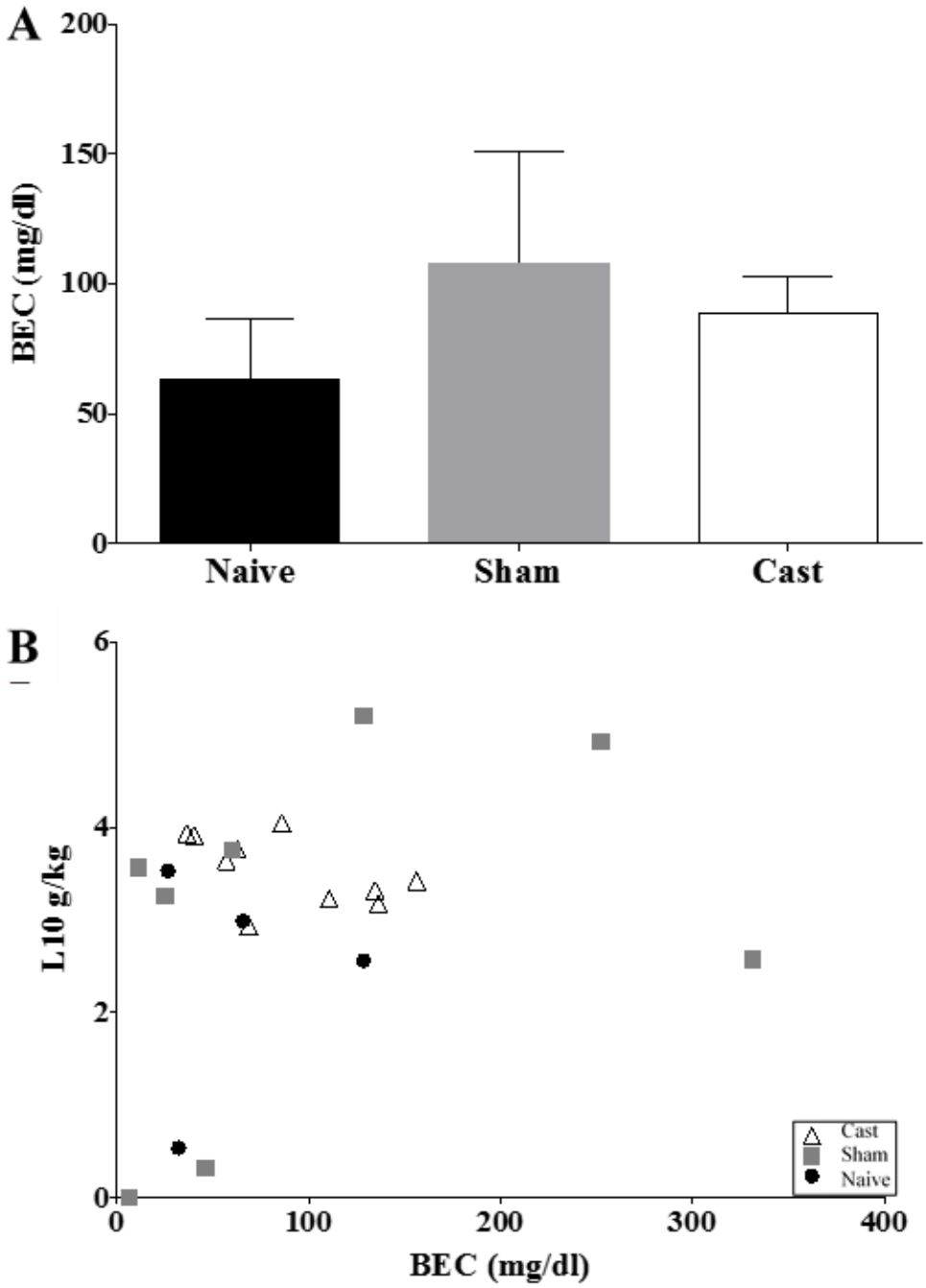

Figure 7. A shows the average (± SEM) blood ethanol content (BEC) in each gonadal condition. B shows the correlation between g/kg alcohol consumed on the final day (L10) and resultant BEC ($r = 0.478$, $p < 0.05$).

Experiment 1B Discussion

Castration did not result in changes to either overall alcohol consumption or consumption in response to exercise restriction, contrary to our hypothesis that testicular hormones would confer some degree of protection against exercise restriction-induced increases in alcohol self-administration. This finding suggests that testicular hormones do not mediate the observation that males are less susceptible to increasing alcohol consumption in response to exercise restriction. All mice, regardless of gonadal condition, increased consumption in response to exercise restriction. Naïve and castrated animals only showed this increase in dose administered, and sham animals only significantly altered consumption in measures of preference.

All groups significantly increased alcohol consumption in response to wheel restriction, but there was a tendency for sham animals to increase their consumption and preference to a greater extent than the naïve or castrated animals. The shams were particularly variable in the dose of alcohol that they administered. It's possible that surgery may produce long-term effect on drinking behavior, as evidenced by the fact that sham animals did not tend to behave identically to naïve animals. Sham surgery may sensitize male animals to the effects of exercise restriction, inducing escalated vulnerability to such aversive stimuli. Because sham animals only tended to differ from naïve and castrated animals, we cannot conclude the presence of any such effects, but future research may address this possibility.

Castration has a slight tendency to increase overall drinking, although this effect was not significant in this study. It is possible that testicular hormones are partially responsible for protecting against vulnerability to high levels of alcohol consumption but require data with greater power. There were no detectable measures in our study to suggest that castration impacted alcohol metabolism. Rachamin and colleagues (1980) demonstrated a testosterone-dependent effect of estrogen on alcohol metabolism, wherein testosterone lowers metabolism relative to females, but either castration or administration of estrogen to male animals increases metabolism rates to that of females. If the tendency for castrated animals to drink slightly more alcohol overall is a true effect, then it may possibly be mediated by such a mechanism; perhaps castrated animals drink more alcohol because they metabolize alcohol slightly faster and therefore need to drink more in order to reach the same level of intoxication as intact mice. We did not measure alcohol dehydrogenase activity in this study, but there does not seem to be evidence to suggest a role of altered alcohol metabolism in these data.

In response to preliminary data suggesting a more robust difference in drinking behavior after castration, we studied the effect of testosterone replacement in castrated animals using an oral hormone replacement paradigm (data not shown). The data from the replacement study did not produce conclusive results, although this could have been related to difficulty determining the correct dose of testosterone to administer orally to replicate normal levels of testosterone. Our final results that show no large effect of testicular hormones on exercise restriction-induced drinking suggest that we wouldn't expect to see any effect of testosterone replacement.

Experiment 2 Introduction

This experiment was intended to investigate the role of β-endorphin in the tendency for females to increase alcohol self-administration in response to exercise restriction. We utilized a genetically modified strain of mice with varying levels of endogenous β-endorphin to investigate this. We hypothesized that the elevated anxiety levels of β-endorphin-deficient animals would enhance sensitivity to exercise restriction-induced alcohol consumption, but that the ability to produce β-endorphin would be necessary for alcohol consumption to effectively self-medicate the stressed state that may accompany the aversive state of wheel restriction. We would expect, then to see animals with diminished, but not absent β-endorphin to exhibit the greatest sensitivity to effects of exercise restriction, as exhibited by increased consumption on days that running wheels are locked. The data presented in Experiment 2 was published in McGonigle et al., 2016.

Experiment 2 Methods

Subjects

Thirty-six naïve female C57BL/6J mice at an average age of 52 days ± 1 day at the beginning of the experiment were used in this experiment. Subjects included 13 B6, 13 βE-HT and 10 βE-KO mice. Housing conditions were consistent before and during the experiment, except that subjects were moved to single housing in TSE Phenomaster Plexiglas cages that contained a food hopper filled with standard mouse chow as well as a

tube of tap water, both available at all times. The cages were also equipped with a running wheel 11cm in diameter (TSE Systems, Bad Homburg, Germany)

Drinking and wheel procedure

Alcohol was provided and running wheels were locked following the same design described in the general methods. The wheels were locked via a mechanical braking device that is controlled by the TSE PhenoMaster program. The TSE PhenoMaster program was intended as our method for collecting both drinking data and running data, but the program generated unreliable and incorrect consumption data. Therefore, the TSE PhenoMaster program measured only running data and drinking was assessed manually, by reading gradations on a 13mL tube with a ball bearing sipper. Twelve mice could be tested at a time in our facility, so the total testing was run in three trials.

RNA isolation and real-time quantitative RT-PCR

Frozen brains were dissected on ice into three regions of interest using the mouse brain matrix (Kent Scientific). The regions of interest included the ventral hippocampus (−2.5 to −4 mm relative to bregma, 2 mm lateral of the midline and 2 mm from the ventral border) and dorsomedial prefrontal cortex (3.0 to1.0 mm relative to bregma and 3 mm from dorsal border) and the amygdala/BNST. For the last region, samples were removed with a 2 mm diameter punch tool according to the brain atlas of Paxinos and Watson (1.0 to −2.5 mm from bregma, 1.5 mm from the midline, and 1 mm from the ventral border). For analysis, each sample was composed of punches from two randomly paired animals of the same genotype in order to obtain sufficient amounts of tissue. Thus,

for example, one sample from the VH contained four dissected tissues – the left and right VH from two genotype-matched subjects. For all quantitative real-time polymerase chain reaction (qPCR) experiments, data were normalized using the corresponding glyceraldehyde-3 phosphate dehydrogenase (GAPDH) mRNA expression because this mRNA species has been shown previously to be a stably expressed reference gene (Rhinn et al., 2008; Taki, Abdel-Rahman, & Zhang, 2014). All assays had similar optimum PCR efficiencies, and all samples were assayed in duplicate during the same procedure. The results are presented as percent differences normalized to gene expression in the B6 group using the ΔΔ Ct relative quantification method (Schmittgen & Livak, 2008).

Each sample tube was immediately homogenized in lysis buffer for RNA extraction. Total RNA was isolated using the Direct-zol RNA MiniPrep Plus (Zymo Research, Irvine, CA, catalog R2072), according to manufacturer's instructions. Fifty ng of total RNA was reverse-transcribed using the iScript Reverse Transcription Supermix (BioRad, Hercules, CA) according to manufacturer's instructions. Synthesized cDNA corresponding to 500 ng of total RNA was used in each qPCR reaction. qPCR was performed using TaqMan FastStart Essential DNA Probes Master Mix (Roche Diagnostics, Indianapolis, IN) according to manufacturer's instructions. PrimeTime® Std qPCR Assays designed by IDT (Integrated DNA Technologies, Coralville, IA) were performed using CRH (NM_205769; forward primer 5′ AGA AAG GAG AAG AGG AAG AAA ACC 3′ and reverse primer 5′ CCG CAG CCG CAT GTT AG 3′), CRH R1 (NM_007762; forward primer 5′ TGC CTT TCC CCA TCA TTG TG 3′ and reverse primer 5′ GCC CTG GTA GAT GTA GTC AGT A 3′), and the reference gene, GAPDH

(NM_0080845′AAT GGT GAA GGT CGG TGT G-3′and reverse primer 5′-GTG GAG TCA TAC TGG AAC ATG TAG 3′) in duplicate on a LightCycler 96 (Roche Diagnostics, Indianapolis, IN).

Enzyme-linked immunosorbent assays (ELISA)

Corticosterone levels were measured using the corticosterone ELISA kit (Enzo Life Sciences, Farmingdale, NY, catalog ADI-900-097), according to manufacturer's instructions. For the corticosterone assay, plasma was diluted 1:40 with assay buffer. The absorbance was read at 405 nm using an iMark microplate reader (BioRad, Hercules, CA). Sample concentrations for corticosterone were calculated from a standard curve using GraphPad Prism software (GraphPad, La Jolla, CA). The sensitivity of the assay was 27 pg/mL with a range of detection up to 20,000 pg/mL. All samples were assayed in duplicate and all samples from the experiment were assayed during a single procedure.

ACTH levels were measured using the ACTH ELISA kit (Enzo Life Sciences, Farmingdale, NY, catalog ENZ-KIT138) according to manufacturer's instructions. For the ACTH assay, plasma was diluted 1:1. The absorbance was read at 450 nm using an iMark microplate reader (BioRad, Hercules, CA). Sample concentrations for ACTH were calculated from a standard curve using GraphPad Prism software (GraphPad, La Jolla, CA). The sensitivity of the assay was 0.46 pg/mL with a range of detection up to 165 pg/mL. All samples were assayed in duplicate and all samples from the experiment were assayed during a single procedure.

Adrenalectomy

Following rapid decapitation, trunk blood was collected and the bodies were stored on ice for a short time. Left and right adrenal glands were then harvested from each mouse and weighed.

Statistical analyses

Data for qPCR were analyzed by one-way ANOVA independently for each brain region and each gene of interest (CRH and CRH-R1) with Bonferroni's multiple comparison *post hoc* tests using Prism 6.1 software (GaphPad, La Jolla, CA).

Experiment 2 Results

One B6 female became ill and abruptly stopped eating, drinking and running in the last few days of the experiment. She was therefore euthanized and her data from the brief period of aberrant behavior was omitted from analyses.

Drinking across the 14-day experimental period was quite variable, reflecting an overall tendency to increase consumption and preference over the test period. This data is presented in daily genotype average g/kg consumption (Fig. 8A) and preference (Fig. 8B). These data are also presented collapsed across experimental period in (C; g/kg and D; preference across the 4-day baseline period and 5 days of either locked or unlocked activity wheels). Repeated measure analyses showed that while genotype did not influence the amount of EtOH consumed across the entire testing period ($F[2,31] = 2.692$, $p > 0.05$) or the three experimental periods ($F[2,33] = 1.995$, $p > 0.05$) there was an increase in drinking over days ($F[13,403] = 15.787$, $p < 0.001$) and across periods ($F[2,66] = 42.209$, $p < 0.001$). However, changes in consumption did not depend upon genotype across either the 14-day experiment ($F[26,403] = 0.745$, $p > 0.05$) or across the three test conditions ($F[4,66] = 0.605$, $p > 0.05$). Preference for the EtOH solution showed much the same pattern, with one exception. In the repeated measure ANOVA there was a main effect of genotype across the entire 14 day experiment ($F[2,30] = 4.548$, $p < 0.05$), but this effect went away when averaged preference in baseline, unlocked, and locked days were each averaged ($F[2,33] = 2.698$, $p > 0.05$). Again, there were main effects of time ($F[13,390] = 5.096$, $p < 0.001$ and $F[2,66] = 6.353$, $p < 0.01$), but no significant interactions ($F[26,390] = 1.206$, $p > 0.05$ and $F[4,66] = 1.710$, $p > 0.05$).

Tukey's *post hoc* analysis demonstrated that βE-HT mice preferred the EtOH solution more than the βE-KO subjects in the daily repeated measure ANOVA ($p < 0.05$).

In studies with appreciably more statistical power, our lab had previously found that female βE-HTs drank more than B6s and βE-KOs (Grisel, et al., 1999; Williams, Holloway, Karwan, Allen, & Grisel, 2007). Collapsing across all days of the test period, we therefore probed for main effects of genotype in one-way ANOVAs for consumption and preference. Though there were no overall effects of genotype on the g/kg of EtOH self-administered (Fig. 9A; $F[2,33] = 2.397$, $p > 0.05$), genotype did influence overall preference for the drug (Fig. 9B; $F[2,33] = 3.496$, $p < 0.05$). Post hoc Tukey HSD revealed that the significant difference existed between the βE-HTs and βE-KOs ($p < 0.05$). Finally, in a direct test of our hypothesis that low endorphin would increase susceptibility to exercise restriction-induced drinking when the wheels were locked, we evaluated the difference in average consumption and preference between locked and unlocked days for each genotype separately (Fig. 9C & D). βE-HTs and βE-KOs increased alcohol consumption in response to exercise restriction, but B6s did not, as evidenced by one-sample *t*-tests comparing the difference scores between locked and unlocked consumption to the null hypothesis, zero (βE-HTs: $t[12] = 3.193$, $p < 0.01$; βE-KOs: $t[9] = 2.577$, $p < 0.05$; and B6s: $t[8] = 1.011$, $p > 0.05$). One-sample *t*-tests revealed no change in preference for any of the genotypes in response to wheel restriction (Fig. 9D; B6 $t[8] = 0.647$, $p > 0.05$; βE-HT $t[12] = 0.836$, $p > 0.05$; βE-KO $t[9] = 0.274$, $p > 0.05$). Only β-endorphin deficient subjects consumed more alcohol on locked than unlocked days.

Constitutive differences in β-endorphin did not affect running wheel behavior. Repeated measures ANOVA comparing average wheel rotations across the three experimental periods showed no overall effect of genotype (Fig. 10 A&B; $F[2,33] = 0.740$, $p > 0.05$) but a main effect of time reflecting a drop in activity on the days with 3 hours of locked wheels (Fig. 10B; $F[1,33] = 4.157$, $p = 0.05$). There was no significant interaction between time and genotype ($F[2,33] = 1.126$, $p > 0.05$).

One-way ANOVA comparing BECs between groups revealed no differences (Fig. 11A: $F[2,32] = 0.563$, $p > 0.05$) though we did observe the expected relationship between EtOH consumed on the final day of the experiment and BEC (Fig. 11B: Pearson correlation $r = 0.667$, 2-tailed $p < 0.001$), and correlations did not significantly differ between genotypes ($p > 0.05$).

Corticosterone levels were also measured from trunk blood collected immediately following the final drinking session. CORT was elevated in βE-HTs compared to either B6s or βE-KOs and these did not differ from each other (Fig. 12A; $F[2,30] = 6.603$, $p < 0.01$, Tukey HSD: B6/ βE-HT $p < 0.01$, βE-KO/ βE-HT $p < 0.05$). Two statistical outliers were removed from the analysis of CORT levels (1 βE-KO, 1 βE-HT). One-way ANOVA investigating genotypic differences in ACTH levels revealed no significant group differences ($F[2,32] = 0.947$, $p > 0.05$; data not shown) but this assay may have been insufficiently sensitive as measures of ACTH detected in our ELISA ranged from 2-65 pg/µl, with an average of 31 ± 2.82 pg/µl. There was a positive correlation between

the g/kg consumed on the last experimental day, and plasma CORT levels (Fig. 12B; Pearson Correlation $r = 0.515$, $p < 0.05$).

Genotype influenced CRH mRNA expression in all brain regions examined (Fig. 13A-C). In the ventral hippocampus, CRH mRNA expression varied significantly (Fig. 13A; $F[2, 14] = 18.67$, $p > 0.05$) and was lower in the B6 mice compared with the βE-KO mice but the CRH mRNA expression in βE-HT mice was not significantly different from the other two genotypes. In the amygdala/BNST CRH mRNA expression also differed overall (Fig. 13B; $F[2,14] = 22.0$, $p < 0.05$) but was similar in the B6 and βE-HT mice which exhibited significantly less CRH mRNA expression compared with the βE-KO mice ($p < 0.05$). In the dorsomedial prefrontal cortex CRH mRNA expression differed ($F[2,14] = 18.31$, $p < 0.05$) in that the βE-KO and B6 mice were similar and βE-HT mice showed lower expression than either of these genotypes ($p < 0.05$). In contrast, there were no genotypic differences in the expression of CRHR1 mRNA in any of the brain regions examined (Fig.13D-F VH: $F[2,14] = 2.049$, $p > 0.05$; Amygdala/BNST: $F[2,14] = 0.076$, $p > 0.05$; PFC: $F[2,14] = 1.472$, $p > 0.05$).

There were significant correlations between dmPFC CRH mRNA and plasma CORT levels (Fig. 14A), demonstrated by a Pearson correlation $r = 0.649$, $p < 0.05$. There was also a relationship between dmPFC CRH mRNA and VH CRH mRNA (Fig. 14B; Pearson correlation $r = 0.487$, $p < 0.05$). In neither of these cases did the correlations significantly differ between genotype.

Though there were no significant differences between the adrenal gland weights across genotypes, there was a tendency, especially in the left adrenal glands, for an inverse relationship between β-E levels and mass. ANOVA of combined left and right adrenals by genotype indicated no effect of genotype on total adrenal mass weight (data not shown; $F[2,20] = 2.421$, $p = 0.114$) or on mass of the left adrenals alone (Fig. 15; $F[2,20] = 3.167$, $p = 0.064$).

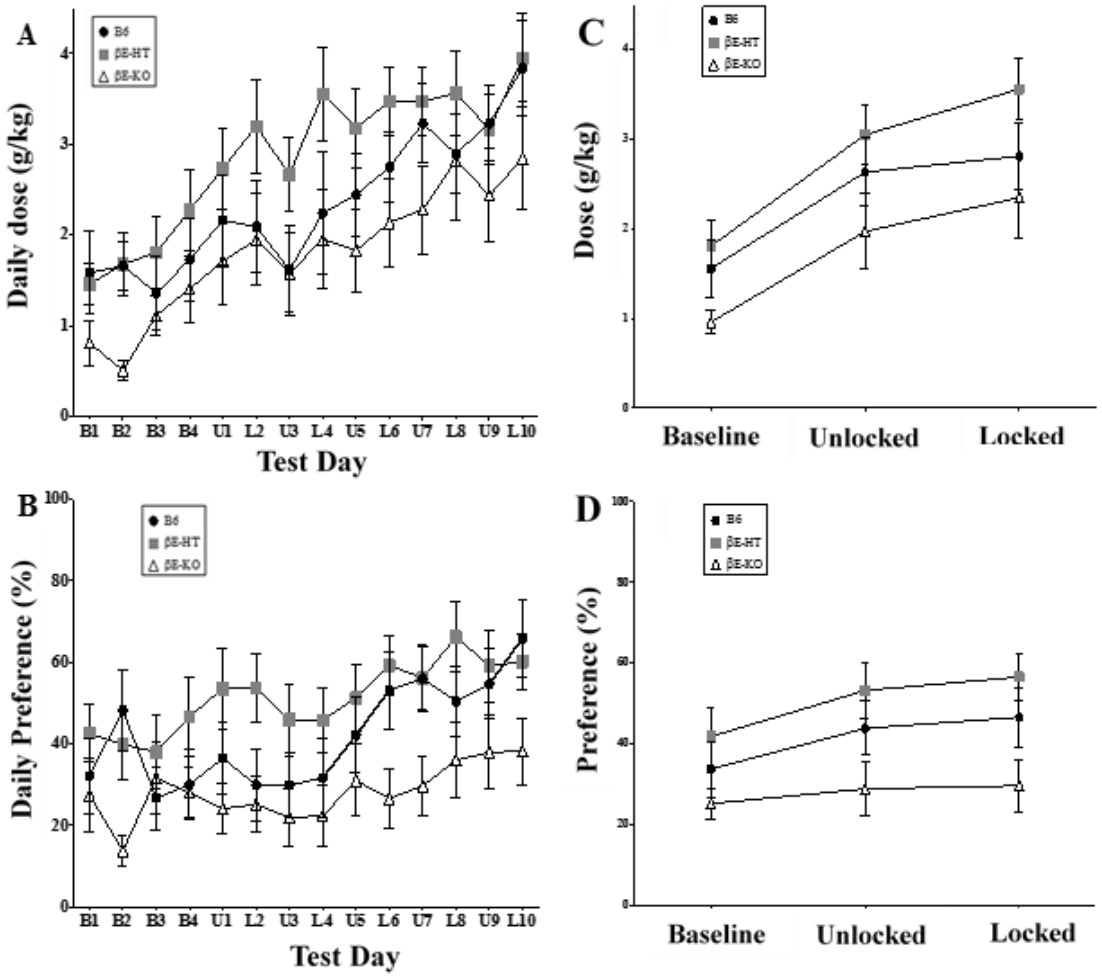

Figure 8. A & C show group means ± SEM for daily consumption and preference and B & D show averaged consumption and preference across the experimental phases of the study. “B” indicates baseline days, “U” indicates unlocked days, “L” indicates locked days.

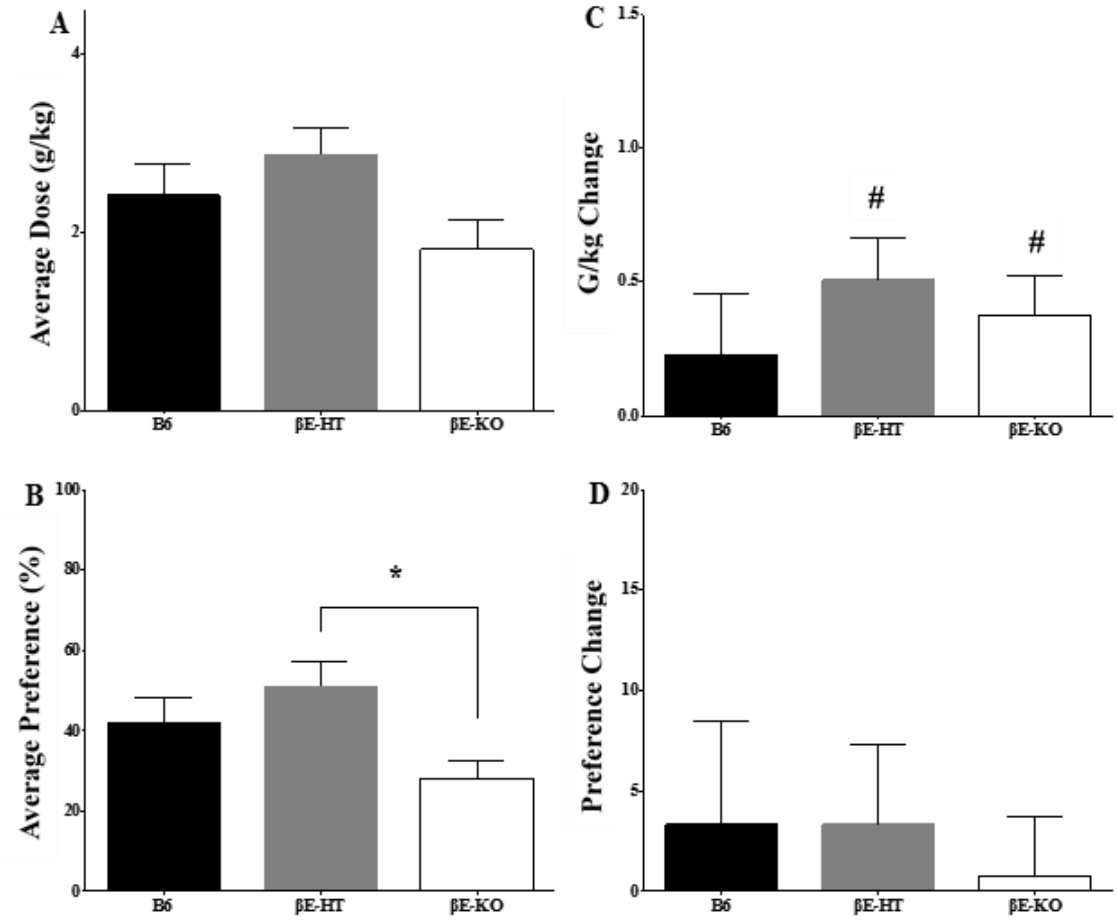

Figure 9. A & B show group means ± SEM for overall alcohol consumption and preference, collapsing across the entire experimental period. C &D show the difference, in consumption and preference, between locked and unlocked days by genotype. * denotes significant difference between βE-HT and βE-KO preference, # denotes significant difference from zero at $p < 0.05$.

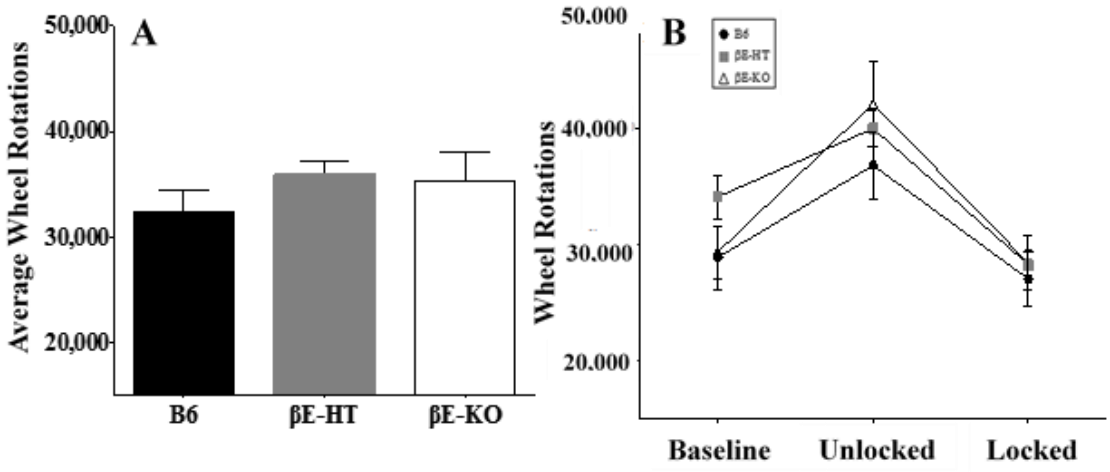

Figure 10. A shows average wheel rotations across the experimental period (genotype means ± SEM) and rotations within each of the experimental phases of the study (B) by genotype.

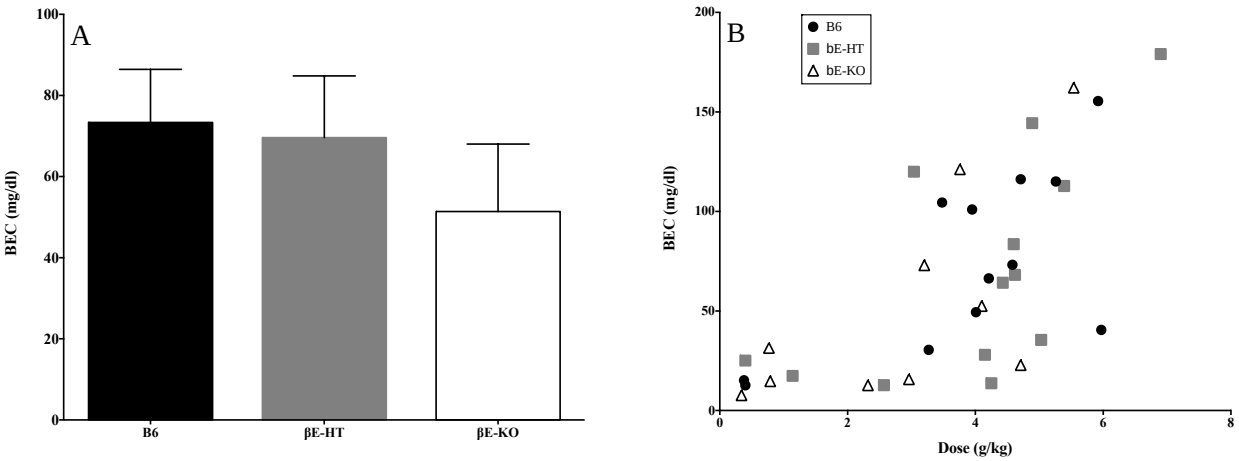

Figure 11. A shows the average (± SEM) blood ethanol content (BEC) in each genotype measured on the final day and B shows the correlation between alcohol consumed on the final day and resultant BEC ($r = .667$, $p < 0.05$).

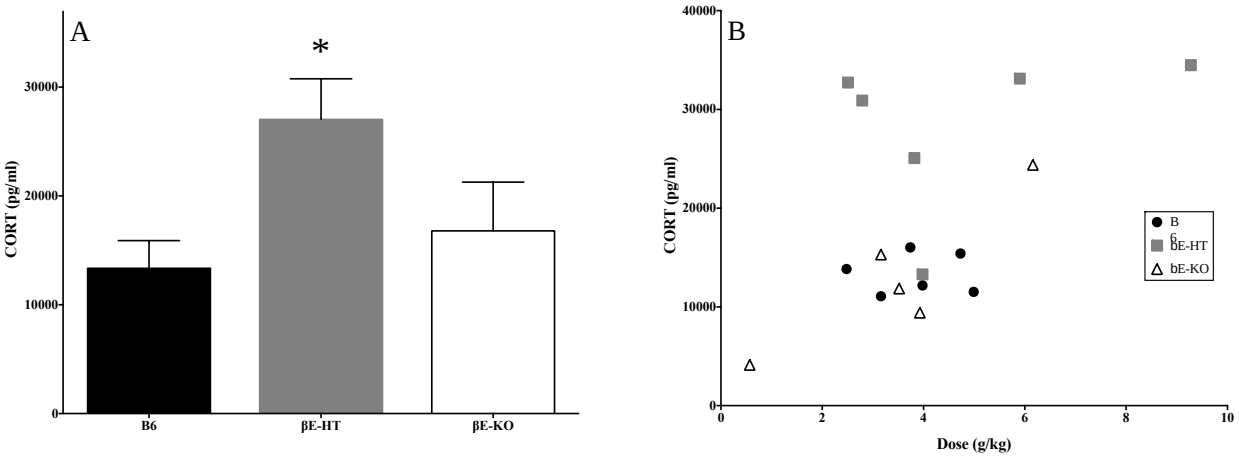

Figure 12. A shows the group means ± SEM for corticosterone levels, and B shows the correlation between alcohol consumed on the final day and corticosterone levels ($r = 0.515$, $p < 0.05$). * denotes significant difference between βE-HT and B6/βE-KO at $p < 0.05$.

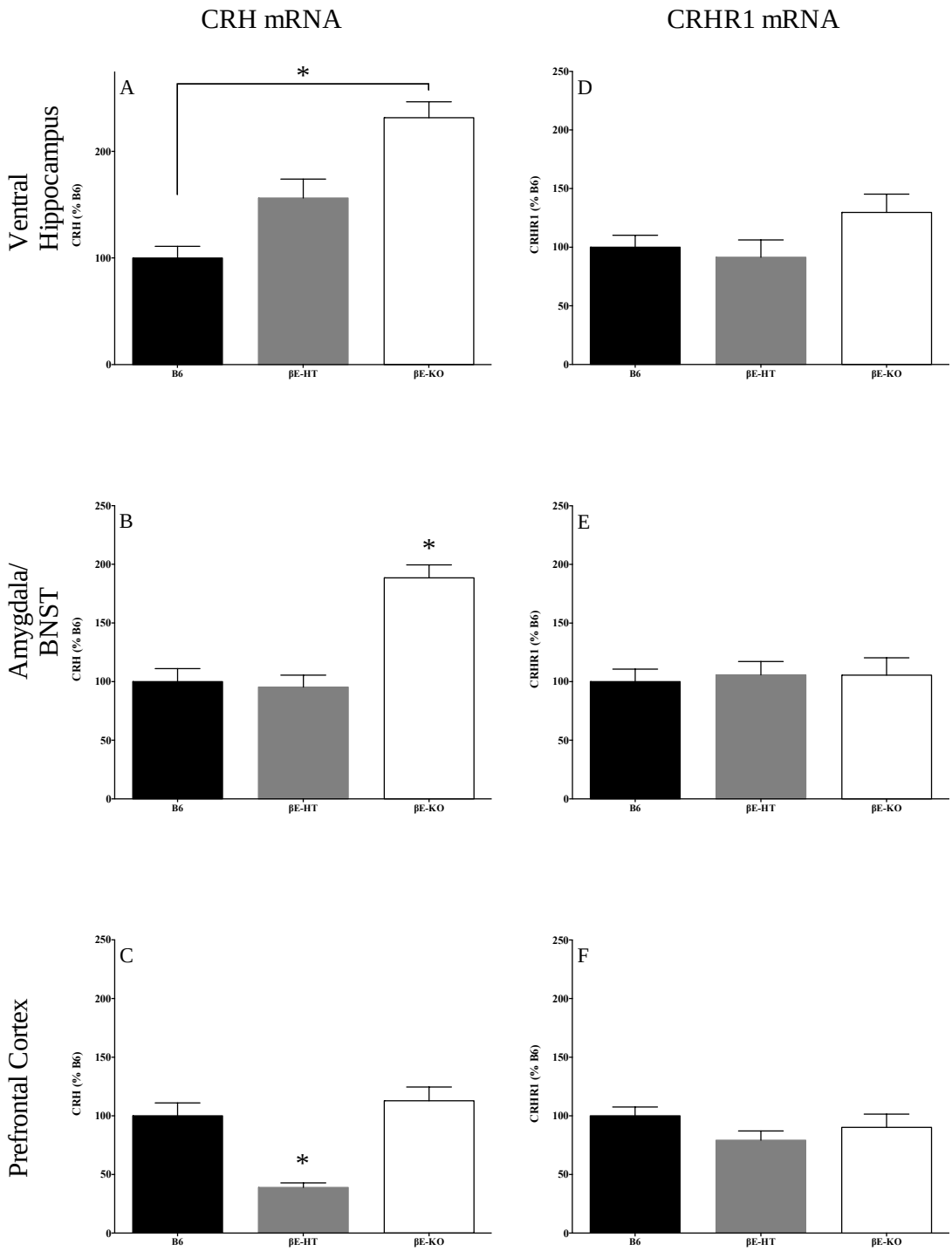

Figure 13. Group means ± SEM for CRH mRNA levels in the ventral hippocampus (A), amygdala/BNST (B), and dorsomedial prefrontal cortex (C). Also shown are group means ± SEM for CRH-R1 mRNA levels in the ventral hippocampus (D),

amygdala/BNST (E), and dorsomedial prefrontal cortex (F). * denotes significance at $p < 0.05$.

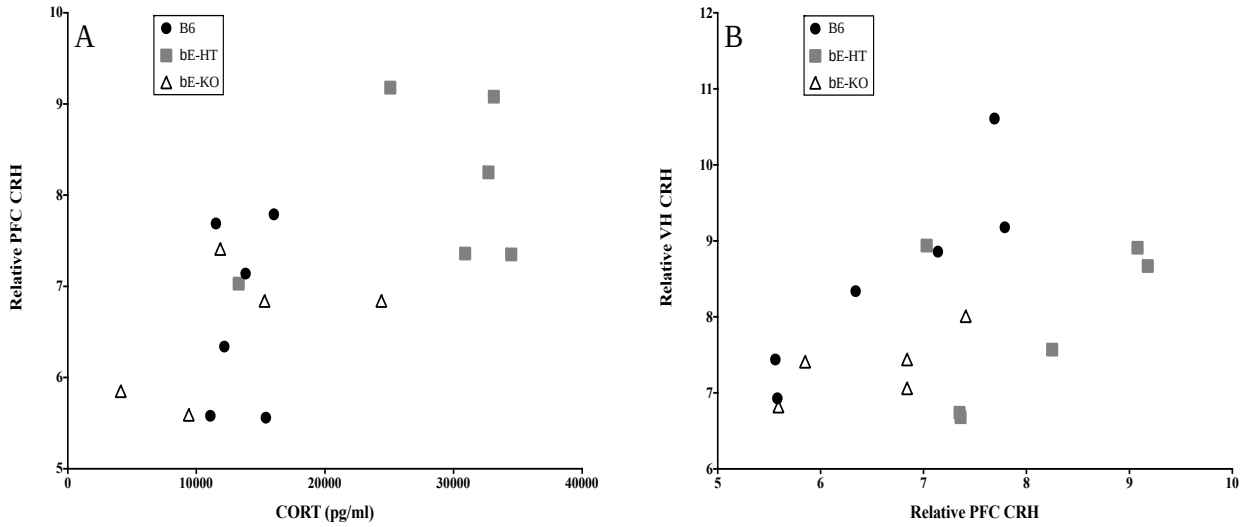

Figure 14. A depicts the correlation between dorsomedial prefrontal cortex CRH mRNA levels and plasma corticosterone levels ($r = 0.649$, $p < 0.05$) and B shows the correlation between CRH mRNA in the dorsomedial prefrontal cortex and the ventral hippocampus ($r = 0.487$, $p < 0.05$).

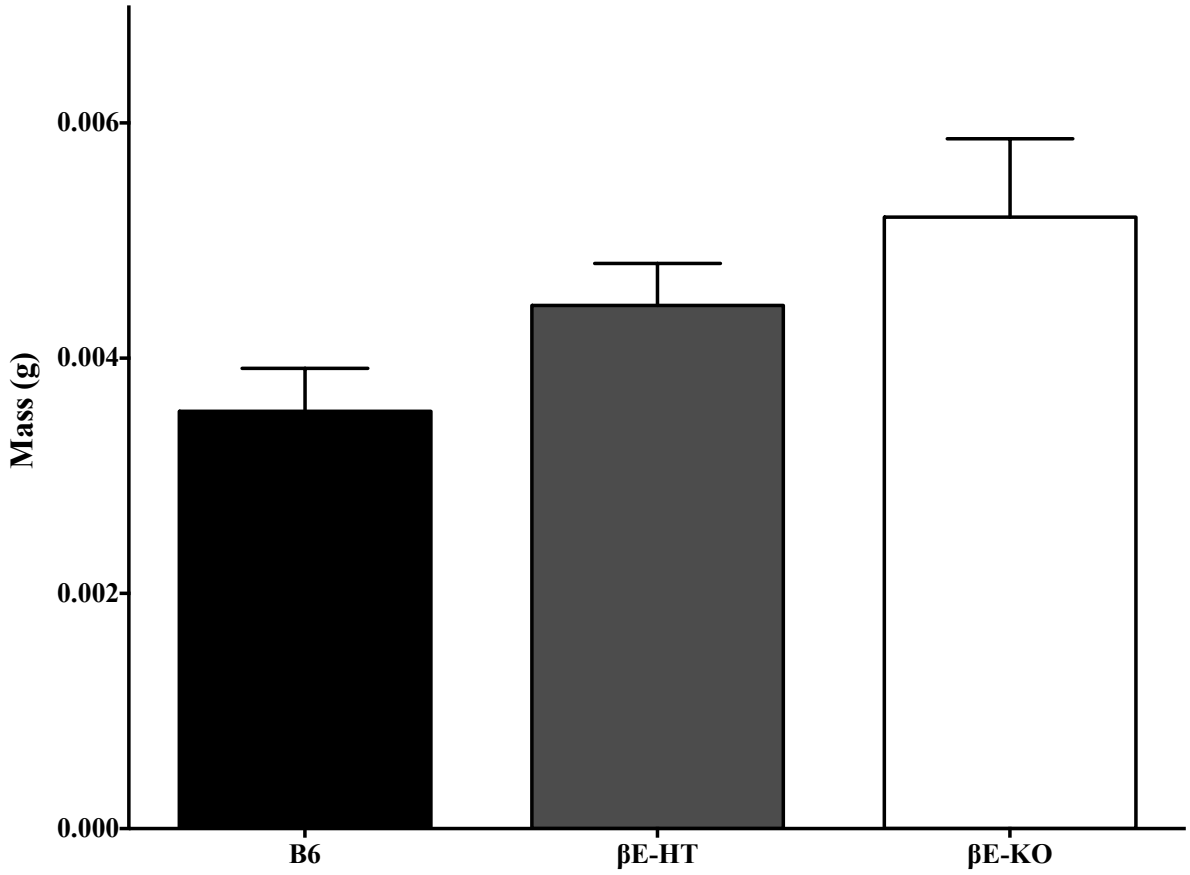

Figure 15. Group means ± SEM for left adrenal gland weights measured after the final day of the experiment.

Experiment 2 Discussion

Our results indicate that low β-endorphin levels are associated with elevated alcohol consumption in response to exercise restriction. We also found increased plasma CORT in transgenic mice deficient of this peptide as well as dysregulation of CRH, compared to B6 controls, consistent with previous research that demonstrated an inverse relationship between stress sensitivity and β-endorphin. Our data, adding to a body both clinical and basic evidence (cf: del Arbol, et al., 1995; Thiagarajan, Mefford, & Eskay, 1989), supports the idea that this opioid peptide influences endocrine and behavioral components of the stress response and contributes to the increased susceptibility for heavy drinking. The variable and moderate self-administration of EtOH evidenced in our experiment adds new insight to a large collection of studies involving the role of stress in alcohol dependence. Moreover, evaluation of low β-endorphin heterozygous mice (βE-HT) enables a nuanced analysis for the role of this peptide beyond what has been shown in earlier studies only comparing βE-KOs and B6s (Mogil, et al., 2000; Racz, et al., 2008) and may better model the human condition.

Stress and the ability to cope with stressful situations have been implicated as causal factors in the development of alcoholism (Bolton, et al., 2006; Brown, et al., 1995; Gianoulakis, et al., 1989). Women are a disproportionately growing subset of the overall population of people with alcohol use disorders (Greenfield, et al., 2010) and also suffer from stress-related psychiatric disorders at about double the rate of men (Kessler, et al., 1993; Marcus, et al., 2005). Therefore, understanding the link between stress susceptibility and the causes of alcoholism is likely to be beneficial for the development

of interventions and treatments in this increasingly at-risk group. In our study, female βE-HTs and βE-KOs, but not B6s increased alcohol consumption in response to exercise restriction, suggesting susceptibility to such aversive stimulus-induced drinking is linked to low β-endorphin levels. Mice with low or absent β-endorphin also display a stronger aversion to novelty-feeding after stress exposure than do B6 controls, suggesting that higher susceptibility that could be linked to a lack of coping skills (Barfield, et al., 2013). Since BECs were correlated with oral consumption, and not influenced by genotype, differences in drinking are likely mediated by pharmacodynamic, rather than pharmacokinetic effects of EtOH. B-endorphin produces its effects by acting on μ, δ, and κ opioid receptors, binding preferentially to the μ receptor (Hallberg & Nyberg, 2003). Subsequent to the development of the β-endorphin knockout model by Rubinstein and colleagues (1996), the model was further characterized to show no differences in opioid receptor binding or expression between the B6 and βE-KO mice (Mogil, et al., 2000). Because there are no differences between opioid receptors between genotypes, we are able to conclude that the effects of β-endorphin deficiency are likely related to downstream targets of β-endorphin. Effects observed in the β-endorphin deficient mice may be regulated by any of these three opioid receptors and receptor agonist or antagonist studies would be vital to furthering the understanding of β-endorphin's mechanism of action.

The effects of β-endorphin on free choice drinking were concomitant with alterations in mRNA for the CRH peptide, but not its receptor, in the three brain regions we checked including the ventral hippocampus (VH), extended amygdala (BNST), and

dorsal medial prefrontal cortex (dmPFC). The VH, among other functions, helps contextualize environmental stimuli and alert other brain areas to stressful situations. The negative correlation between CRH mRNA expression levels and β-endorphin in the VH mirrors differences previously seen in anxiety-like behavior, with mice completely lacking β-endorphin having the most anxious phenotype and highest levels of CRH mRNA (Barfield, et al., 2010; Grisel, et al., 2008). Such data suggest chronic upregulation of the HPA axis associated with β-endorphin deficiency (Rubinstein, et al., 1996). Our lab had previously found enlarged adrenal glands in β-endorphin-deficient animals and although there were not significant effects of genotype on adrenal weight in the current study, the same tendency was evident in this smaller and younger cohort of subjects (Grisel, et al., 2008). Notably, we did not observe genotypic differences in ACTH levels, although measured levels were low compared to expected values, suggesting that perhaps our assay wasn't sufficiently sensitive. The VH sends glutamatergic projections to the basolateral amygdala, linking the excitation of these two regions (Stamatakis, et al., 2014). This may help explain why the βE-KOs have higher levels of CRH in both the VH and Amygdala/BNST compared to B6s.

The amygdala and BNST also play a major role in assessing stressors and helping to coordinate anxiety-related behaviors. The amygdala is activated by stress and has glutamatergic projections that lead to both the medial prefrontal cortex and the BNST. The BNST, located between the amygdala and the nucleus accumbens, connects stress and reward centers of the brain. Our samples combined the BNST and amygdala, so we are not able to parse specific contributions of these areas, but nonetheless found that βE-

KOs that preferred and consumed the least EtOH overall showed about twice the CRH mRNA expression in this limbic area. This elevated CRH expression may be a possible mechanism through which the Rubinstein βE-KO model has elevated anxiety-related behaviors (Barfield, et al., 2010; Barfield, et al., 2013; Grisel, et al., 1999). Though we did not hypothesize that βE-KOs mice would self-medicate basal stress with EtOH (Grisel, et al., 1999), we found that mice entirely deficient in the peptide did increase intake on locked days relative to days when the wheels were unlocked. Lower CRH in βE-HTs may indicate successful self-medication by EtOH consumption in response to aversive stimuli. The mechanism for this may include EtOH-induced CORT release inhibiting subsequent CRH production. Hyperexcitability of the BNST has been seen in chronic drug use and is associated with increased stress susceptibility (Silberman & Winder, 2013; Stamatakis, et al., 2014). It may be that CRH in the BNST and amygdala remains overactive and unregulated in βE-KOs, but in the βE-HTs, possibly mediated by a drinking-induced synthesis of β-endorphin, can be regulated. The BNST sends CRH to the PVN of the hypothalamus, exciting the region further and leading to higher activation of the stress axis, including increased release of ACTH and CORT (Myers, et al., 2015).

The dmPFC contributes to processing of emotionally salient information. It is possible that significantly lower levels of CRH in the dmPFC for the βE-HTs is also linked to effective self-medication of high aversion sensitivity by consumption of EtOH. Because CORT acts in the dmPFC to inhibit the HPA axis via GABAergic projections to the posterior nucleus of the hypothalamus (Myers, et al., 2015) the low CRH message in βE-HTs may account for relatively higher levels of plasma CORT in these subjects. This

idea is supported by the overall positive correlation between plasma CORT and dmPFC CRH mRNA, but it remains possible that high drinking has direct effects on changes in mRNA and CORT.

It might have been assumed that mice lacking β-endorphin would be less active on the running wheels, an expectation related to the proposed role of β-endorphin in producing the "runner's high" (Dinas, Koutedakis, & Flouris, 2011). However, we did not see any differences in running as a function of genotype. Recently, endocannabinoids were implicated in the euphoric effects of running (Fuss, et al., 2015), but locomotor activity is no doubt mediated by a constellation of genetic, chemical and anatomical influences. Because there were no differences in wheel running related to genotype however, we are able to conclude that our results were not impacted by differences in the salience of the activity wheel as a function of β-endorphin.

Previous investigation of the effect of restricted wheel access on drinking in our lab (Piza-Palma, et al., 2014), using a large group of subjects, showed that female but not male B6 mice increased alcohol consumption in response to exercise restriction, and therefore we did not include males in the present study. However, it remains possible that low, in contrast to absent, β-endorphin would alter the susceptibility to exercise restriction-induced alcohol consumption and shift the reward sensitivity curve in male mice, as it did in females. Ongoing studies are aimed at elucidating sex differences in the response to restricted wheel access. A limitation of the present study is that we did not assess endocrine and CRH related measures in alcohol naïve animals, and so we are unable to determine whether endocrine and CRH expression differences between groups

are directly attributable to differences in β-endorphin or a result of alcohol exposure interacting with constitutive differences in the opioid. Nonetheless, because we employed a between subject (genotype) design, our data support the overarching hypothesis that low, but perhaps not absent, β-endorphin increases susceptibility to the negatively-reinforcing effects of EtOH. Because drinking patterns did not differ between βE-HT/ βE-KO and B6 animals, these effects of diminished β-endorphin may be small in magnitude and perhaps require larger sample sizes to detect these nuanced differences.

Experiment 3 Introduction

This experiment was intended to investigate the interaction of testicular hormones and β-endorphin in alcohol consumption following restriction of access to voluntary exercise. We utilized a β-endorphin knockout model combined with castration to investigate the possibility of such an interaction. Previous research in our lab suggested a testosterone-dependent effect of β-endorphin on sensitivity to the loss of righting reflex effect of alcohol administration. In our model measuring susceptibility to self-administer alcohol in response to exercise restriction, elevated vulnerability is demonstrated by higher consumption on days when homecage running wheels are locked before and during alcohol availability.

Experiment 3 Methods

Subjects

Thirty-eight alcohol-naïve male mice with a C57BL/6J (B6) genetic background at an average age of 81 days ± 2 days at the beginning of the experiment were used in this experiment. Subjects included 6 sham B6, 4 castrated B6, 6 sham βE-HT, 7 castrated βE-HT, 7 sham βE-KO, and 8 castrated βE-KO mice. Housing conditions were consistent before and during the experiment, except that subjects were moved to single housing in cages that contained a food hopper filled with standard mouse chow and two tubes of water (25 mL capacity with ball-bearing sipper), available at all times. The cages were also equipped with a 4.1” x 6.1” x 6.0” running wheel (Med Associates Inc., Vermont).

Surgical procedure

Mice underwent castration surgeries as detailed in the general methods after 50 days of age. The average age of mice at the time of surgery, was 68 days ± 2 days old.

Drinking and wheel procedure

Alcohol was provided and running wheels were locked following the same design described in the general methods. The water and alcohol for this experiment were provided in 25 mL graded tubes with ball-bearing tips. During alcohol availability, one water tube was removed and replaced with an identical tube that contained the 20% ethanol solution. The side of alcohol presentation was alternated to prevent mice from developing a side preference for drinking. The model of running wheels used in this experiment were locked manually by insertion of a small pin between the wheel and its casing, which was then removed at the end of alcohol availability. 15 mice could be tested at a time in our facility, so the total testing for experiment 3 was run in three trials over the course of four months.

Experiment 3 Results

Repeated-measure ANOVA of daily g/kg consumption (Fig. 16A & B) by condition revealed a tendency for condition to affect g/kg, with knockouts consuming the lowest amounts of alcohol ($F[5,27] = 2.500$, $p = 0.055$). There was also a main effect of time, reflecting a tendency for alcohol consumption to increase over the course of the ten day experiment ($F[9,243] = 2.411$, $p < 0.05$). This escalation in consumption over the experiment did not change differently across conditions ($F[45,243] = 1.131$, $p > 0.05$). Because our hypothesis was not necessarily comparing all conditions directly to one another, but rather asking whether the effect of castration depended on genotype, we also evaluated each measure within gonadal condition, and within genotype. There was still a main effect of time for all B6 animals ($F[9,72] = 2.176$, $p < 0.05$), but there was no effect of gonadal condition on B6 animals in daily consumption ($F[1,8] = 0.943$, $p > 0.05$). Likewise, the change in consumption over time did not vary with condition ($F[9,72] = 1.688$, $p > 0.05$). In heterozygous mice, there was no effect of time, gonadal condition, or an interaction of time and condition (time: $F[9,72] = 1.392$, $p > 0.05$; condition: $F[1,8] = 2.656$, $p > 0.05$; interaction: $F[9,72] = 0.836$, $p > 0.05$]). In βE-KO mice, there were again no effects of time, condition, or an interaction (time: $F[9,99] = 0.667$, $p > 0.05$; condition: $F[1,11] = 0.491$, $p > 0.05$; interaction: $F[9,99] = 1.524$, $p > 0.05$). When examining the effect of genotype on behavior within either the castrated or the gonadal condition, there likewise were no significant effects in the repeated-measure ANOVA. There was no main effect of time on drinking in castrated mice (Fig. 16B; $F[9,126] = 1.458$, $p > 0.05$). Also there was no effect of genotype on drinking in castrated mice and no interaction between

genotype and time in the castrated mice (genotype: $F[2,14] = 2.145$, $p > 0.05$; interaction: $F[18,126] = 0.988$, $p > 0.05$). Similarly, there were no main effects of time and genotype, or an interaction between factors in sham mice (Fig. 16A; time: $F[9,117] = 1.645$, $p > 0.05$; condition: $F[2,13] = 2.039$, $p > 0.05$; interaction: $F[18,117] = 1.530$, $p > 0.05$).

Repeated-measure analysis of the daily preference data (Fig. 16C & D) showed very similar results, both overall, and within genotypes and conditions. Overall by condition, there was a main effect of time ($F[9,180] = 2.317$, $p < 0.05$), but no effect of condition ($F[5,20] = 1.083$, $p > 0.05$) or an interaction between time and condition ($F[45, 180] = 0.948$, $p > 0.05$). Examination of the effect of castration within each genotype only resulted in a significant effect of time in heterozygous animals ($F[9,63] = 2.148$, $p < 0.05$). In heterozygous mice, there were no effects of condition ($F[1,7] = 0.662$, $p > 0.05$) or a significant interaction between time and condition ($F[9,63] = 0.824$, $p > 0.05$). There were no effects of daily preference in B6 animals (time: $F[9,45] = 1.122$, $p > 0.05$; condition: $F[1,5] = 0.05$, $p > 0.05$; interaction: $F[9,45] = 0.714$, $p > 0.05$). Neither were there effects of daily preference in βE-KO animals (time: $F[9,72] = 1.145$, $p > 0.05$; condition: $F[1,8] = 2.038$, $p > 0.05$; interaction: $F[9,72] = 1.048$, $p > 0.05$). When examining the effect of genotype within each gonadal condition, the only significant effect was again an effect of time in the sham animals (Fig. 16C; $F[9,63] = 2.288$, $p < 0.05$). There were not an effect of genotype or an interaction with condition in these animals (genotype: $F[2,7] = 1.005$, $p > 0.05$; interaction: $F[18,63] = 1.078$, $p > 0.05$). There were no effects in daily preference in castrated animals (Fig. 16D; time: $F[9,117] =$

0.760, $p > 0.05$; genotype: $F[2,13] = 0.860$, $p > 0.05$; interaction: $F[18,117] = 1.006$, $p > 0.05$).

In order to evaluate the effect of overall drinking behaviors, regardless of wheel availability, we averaged together the drinking of the ten experimental days to produce a measure of average consumption (g/kg and preference). There is no significant impact of overall condition on total g/kg consumption, revealed by one-way ANOVA ($F[5,31] = 2.184$, $p > 0.05$). There is also no effect of gonadal condition within any of the genotypes (B6, Fig. 17A: $F[1,8] = 0.943$, $p > 0.05$; βE-HT, Fig. 17B: $F[1,10] = 2.377$, $p > 0.05$; βE-KO, Fig. 17C: $F[1,13] = 0.037$, $p > 0.05$). Similarly, within gonadal conditions there was no effect of genotype (Sham: $F[2,15] = 3.2328$, $p > 0.05$; Castrated: $F[2,16] = 0.949$, $p > 0.05$). One-way ANOVA revealed the same pattern of results for total preference. There was no effect of overall condition ($F[5,31] = 0.621$, $p > 0.05$). Within each genotype there was no impact of castration (B6, Fig. 17D: $F[1,8] = 0.124$, $p > 0.05$, βE-HT, Fig. 17E: $F[1,10] = 0.00$, $p > 0.05$, βE-KO, Fig. 17F: $[1,13] = 0.015$, $p > 0.05$). Genotype also did not cause total preference within the mice of the same gonadal conditions to differ (Sham: $F[2,15] = 1.693$, $p > 0.05$; Castrated: $F[2,16] = 0.295$, $p > 0.05$).

Susceptibility to drinking in response to exercise restriction in our paradigm is measured by the increase, in alcohol consumption in response to imposition of a locked running wheel. By subtracting average consumption on unlocked days from average consumption on locked days, we get a change score for the amount of exercise restriction-induced increase in consumption. One-way ANOVA of this g/kg change score (Fig. 18A & B) revealed no effect of overall condition on exercise restriction-induced

alcohol consumption ($F[5,31] = 0.654$, $p > 0.05$). Within each genotype, castration also did not change sensitivity to wheel restriction (B6: $F[1,8] = 0.01$, $p > 0.05$; βE-HT: $F[1,10] = 0.029$, $p > 0.05$; βE-KO: $F[1,13] = 0.015$, $p > 0.05$). Within each gonadal condition, genotype also did not produce significant effects on exercise restriction-induced alcohol consumption (Sham: $F[2,15] = 0.629$, $p > 0.05$; Castrated: $F[2,16] = 1.105$, $p > 0.05$). The same pattern of results was observed after running one-way ANOVA on preference change, as well (Fig. 18C & D). There was no effect of overall condition ($F[5,31] = 0.945$, $p > 0.05$). Within each genotype, castration also did not change preference of alcohol when wheels were locked (B6: $F[1,8] = 0.045$, $p > 0.05$; βE-HT: $F[1,10] = 0.153$, $p > 0.05$; βE-KO: $F[1,13] = 0.947$, $p > 0.05$). Within each gonadal condition, genotype also did not produce significant effects (Sham: $F[2,15] = 1.356$, $p > 0.05$; Castrated: $F[2,16] = 0.606$, $p > 0.05$).

There were no sham groups that displayed a significant increase in alcohol g/kg consumption in response to locked wheels (Fig. 18A; S-B6: $t[5] = 1.793$, $p > 0.05$; S-HT: $t[4] = 0.166$, $p > 0.05$; S-KO: $t[6] = 0.116$, $p > 0.05$). There were also no significant increases for castrated βE-HTs (Fig. 18B; $t[6] = 1.605$, $p > 0.05$) or castrated βE-KOs ($t[7] = -0.069$, $p > 0.05$), although castrated B6s did significantly increase g/kg consumption on locked days ($t[5] = 2.271$, $p = 0.05$). There also were no significant increases for sham groups in preference (Fig. 18C; S-B6: $t[5] = 0.462$, $p > 0.05$; S-HT: $t[4] = 1.677$, $p > 0.05$; S-KO: $t[6] = -0.729$, $p > 0.05$). There were no significant increases for castrated B6 (Fig. 18D; $t[3] = 0.665$, $p > 0.05$) or castrated βE-KO ($t[7] = 0.621$, $p >$

0.05), although castrated βE-HTs did significantly increase from zero ($t[6] = 2.651$, $p < 0.05$).

Total running behavior, averaged across the experimental period, differed significantly across conditions (Fig. 19; $F[5,28] = 4.205$, $p < 0.01$). *Post hoc* Tukey HSD revealed that βE-HT castrated mice drink significantly less than βE-KO shams ($p = 0.05$) and less than βE-HT shams ($p = 0.045$). There was no effect of castration on running in the B6s ($F[1,7] = 1.188$, $p > 0.05$), but there was an effect of castration in both other genotypes – castration significantly reducing overall running (βE-HT: $F[1,9] = 16.175$, $p < 0.01$; βE-KO: $F[1,12] = 11.164$, $p < 0.01$). There was no effect of genotype within each gonadal condition (Sham: $F[2,13] = 0.124$, $p > 0.05$; Castrated: $F[2,15] = 0.793$, $p > 0.05$).

There were no differences between BECs between conditions (Fig. 20A; $F[5,31] = 0.561$, $p > 0.05$). There were also no effects of castration in any genotype (B6: $F[1,8] = 0.00$, $p > 0.05$; βE-HT: $F[1,10] = 2.798$, $p > 0.05$; βE-KO: $F[1,13] = 0.598$, $p > 0.05$). Within each gonadal condition, there were no differences between the genotypes (Sham: $F[2,15] = 0.351$, $p > 0.05$; Castrated: $F[2,16] = 0.224$, $p > 0.05$). BECs correlated well with consumption on the final day of the experiment, as expected (Fig. 20B; Pearson correlation $r = 0.506$, $p < 0.005$).

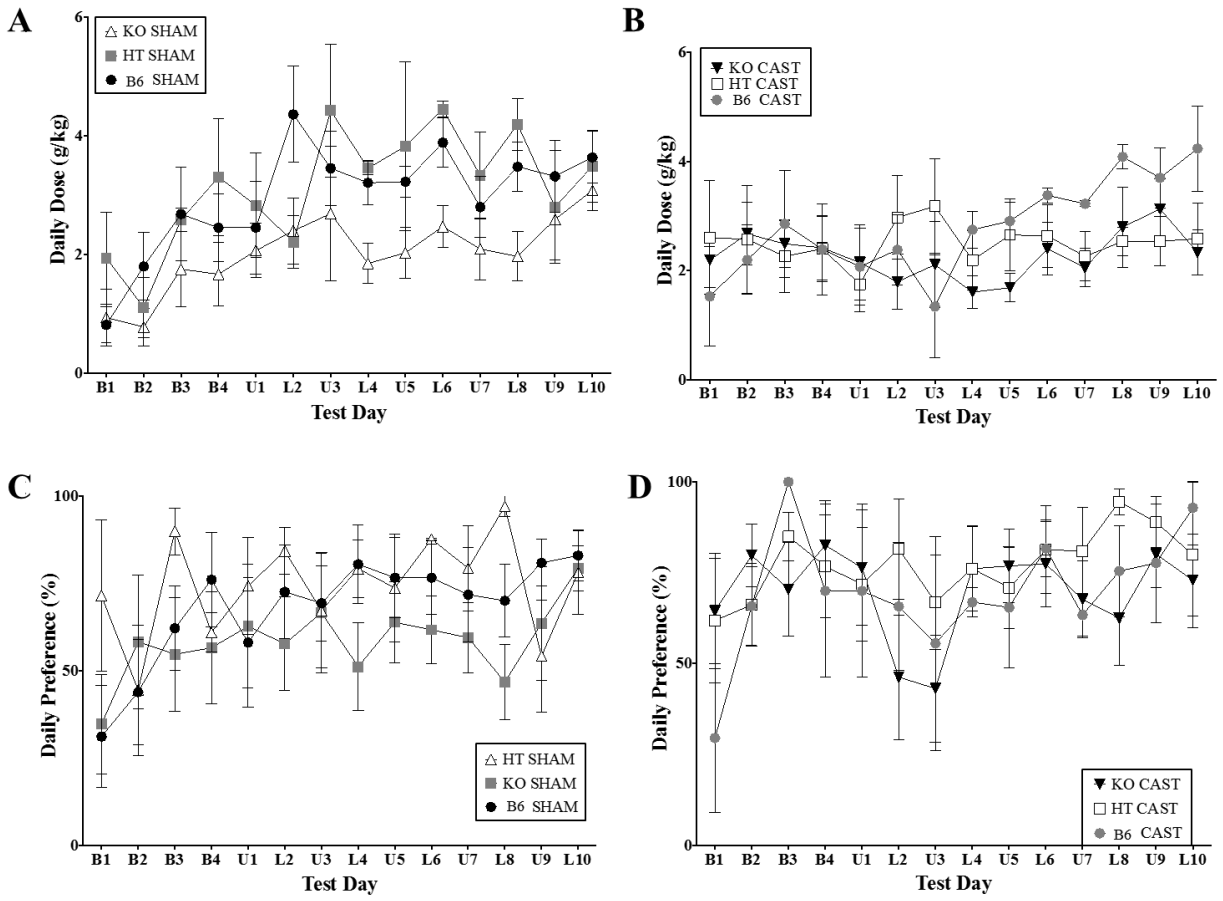

Figure 16. A & B show group means ± SEM for daily g/kg consumption by genotype for sham and castrated conditions. C & D show group means ± SEM for daily preference by genotype for sham and castrated conditions. “B” indicates baseline days, “U” indicates unlocked days, “L” indicates locked days.

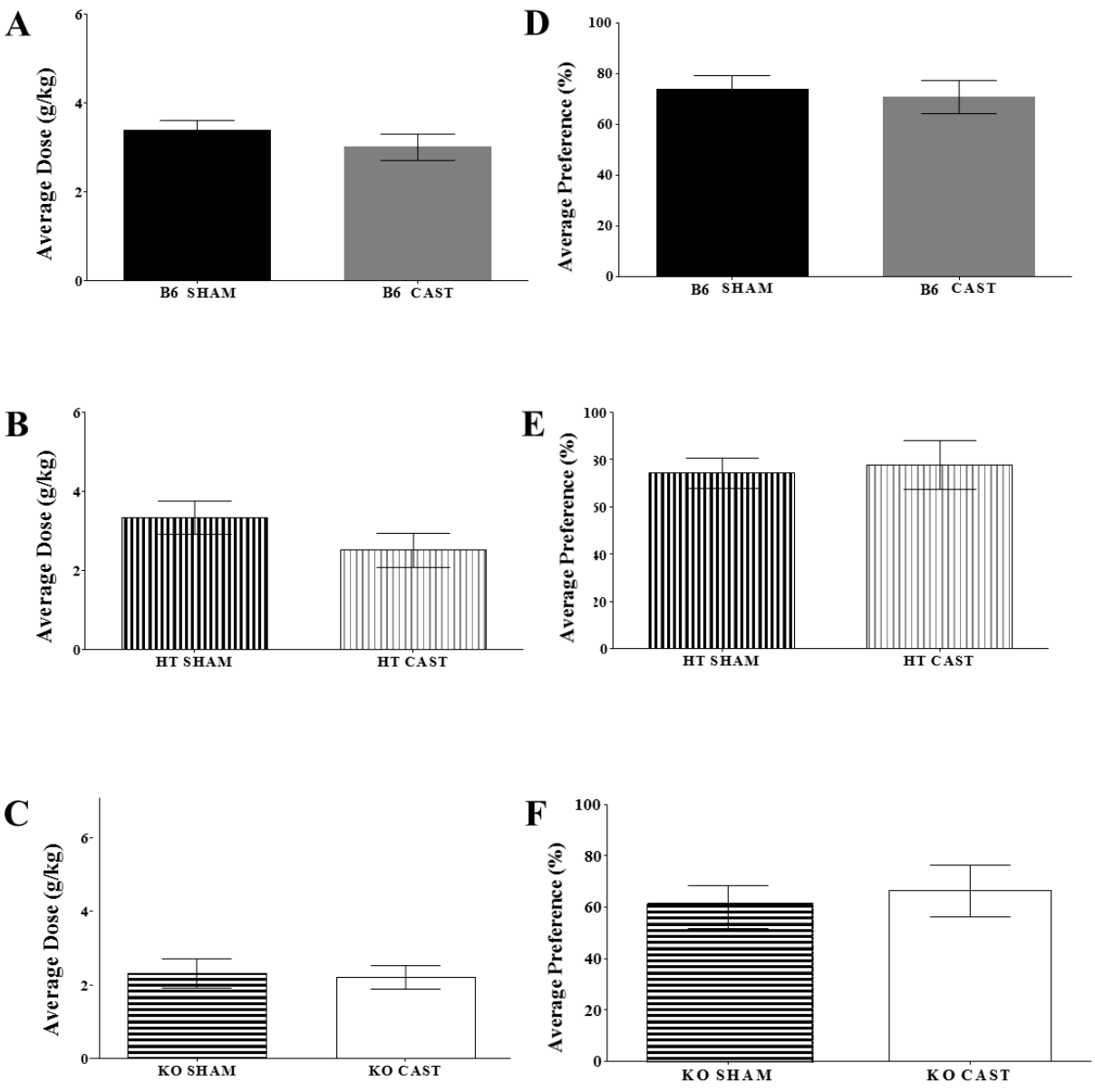

Figure 17. A-C show group means ± SEM for averaged consumption across 10-day experimental period by gonadal condition by genotype. D-F show group means ± SEM for averaged preference across 10-day experimental period by gonadal condition by genotype.

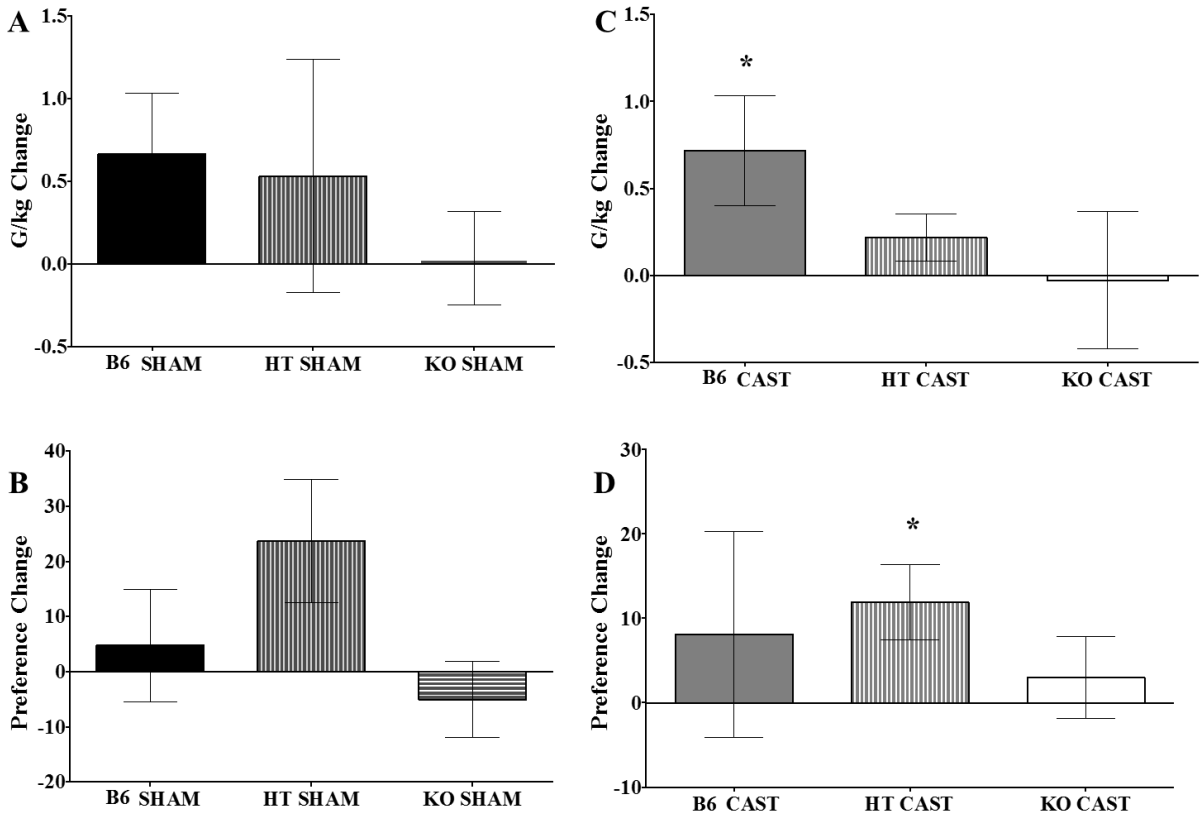

Figure 18. A & B show the difference in consumption (group average ± SEM) between averages of locked and unlocked days by genotype for sham and castrated animals. C & D show the difference in preference (average ± SEM) between averages of locked and unlocked days by genotype for sham and castrated animals. *denotes significance from zero at $p < 0.05$.

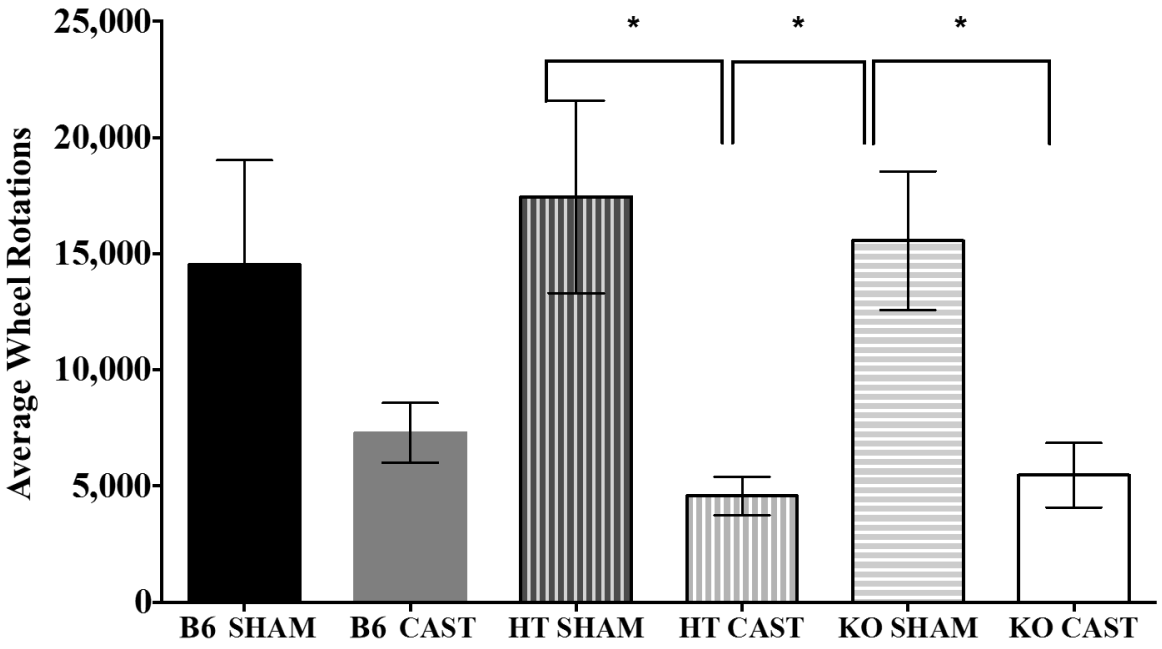

Figure 19. Group means (± SEM) for daily wheel rotations, collapsed across all days of the experiment. *denotes significance between indicated groups at $p < 0.05$.

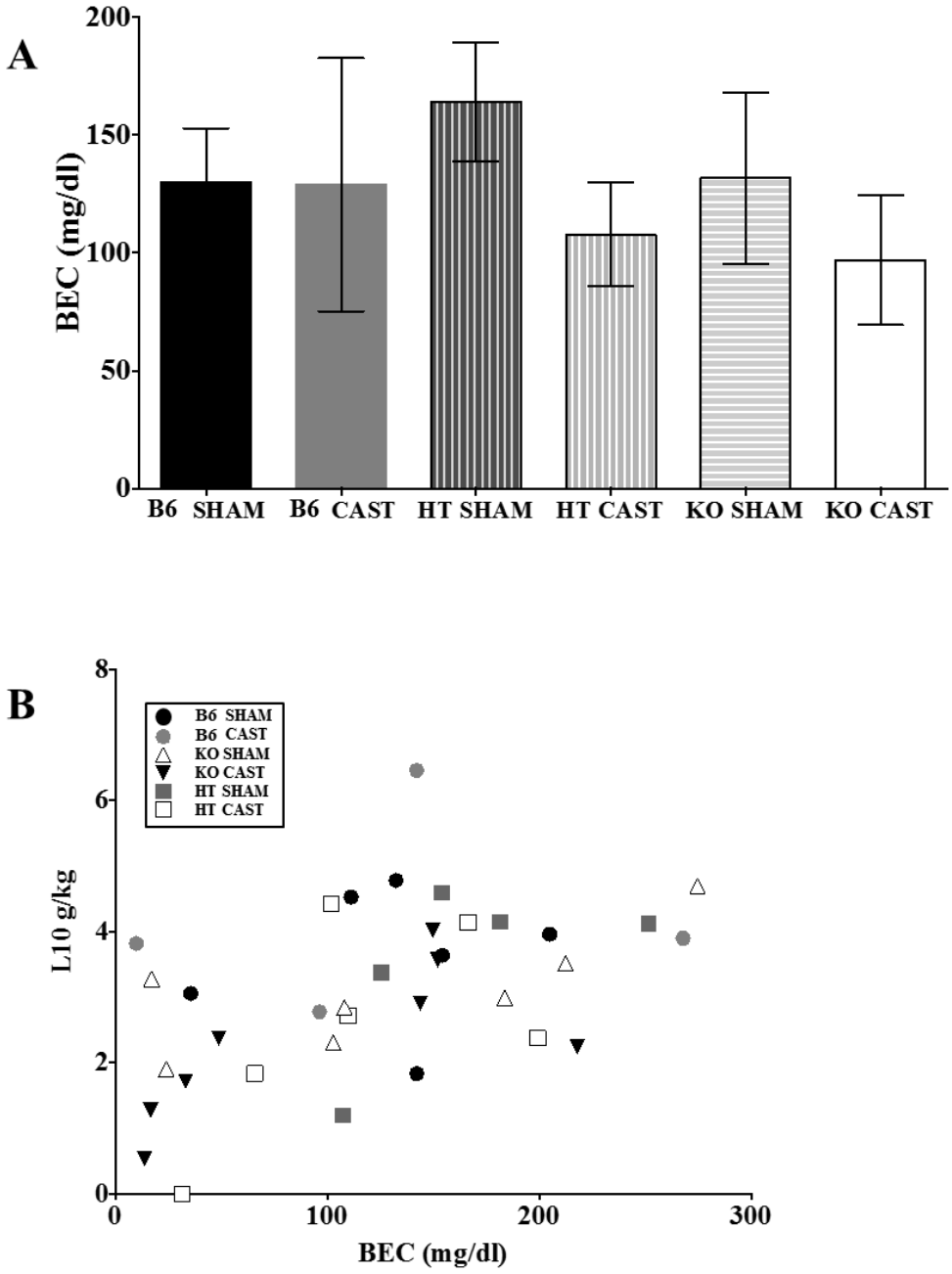

Figure 20. A shows the average (± SEM) blood ethanol content (BEC) in each gonadal condition. B shows the correlation between alcohol consumed on the final day (L10) and resultant BEC ($r = 0.506$, $p < 0.005$).

Experiment 3 Discussion

Building on the hypothesis that β-endorphin plays a mediating role in exercise restriction-induced alcohol consumption, we investigated the interaction between testicular hormones and the levels of β-endorphin. We found no evidence of a β-endorphin interaction with testosterone either on overall consumption or in alcohol consumption elevation following exercise restriction.

Individual differences in alcohol consumption produce particularly variable data, so our small sample sizes made analysis of the interaction between testosterone and β-endorphin challenging. Our lab will conduct at least two more runs of animals in this experiment throughout the next several months in order to increase the sample size of each condition to a minimum of 10 animals. Because of the exploratory nature of this study, we analyzed the data by overall condition, by genotype, and by gonadal condition in all cases. Our current data do not show any significant interactions between β-endorphin and testicular hormones through these analyses, but it is possible that significant relationships will emerge with additional animals that will reveal interactions between β-endorphin and testicular hormones.

Our results suggest that there is no interaction between β-endorphin and testicular hormones in exercise restriction-induced alcohol consumption. There was a strong trend, nearing significance, for sham βE-KOs to drink less alcohol on average than sham βE-HTs. If this trend becomes significant with the addition of more animals in each condition, it would indicate that the degree of β-endorphin deficiency has important

implications for risk of higher alcohol consumption, but only in the context of having normal testicular hormone production. That this effect of β-endorphin deficiency is dependent upon levels of testicular hormones has been suggested previously in the context of sensitivity to loss of righting reflex after administration of a high intra-peritoneal dose of alcohol (Grisel, unpublished). With additional animals, the relationship between testicular hormones and β-endorphin as they relate to alcohol consumption following an aversive state, such as running wheel restriction will become clearer, although the current data does not strongly suggest what the nature of these relationships may be.

Susceptibility to elevating alcohol consumption in response to wheel restriction was only observed in castrated groups in this study. Castrated B6 mice and castrated βE-HT mice increased either g/kg consumption or preference in response to exercise restriction. Following our previous study (1B) that examined the role of testicular hormones in exercise restriction-induced drinking, we would expect that with more animals, the sham B6 animals would also display a significant increase in drinking behaviors on locked days, compared to unlocked days. Should this be the finding with larger sample sizes, it would indicate that β-endorphin deficiency coupled with testosterone possibly protects against elevated alcohol consumption in response to exercise restriction. βE-KO mice likely did not display exercise restriction-induced increases in alcohol consumption due to an inability to effectively self-medicate the aversive state with alcohol consumption when β-endorphin is not present. While behaviorally we did not observe this pattern in the female β-endorphin model, there was

evidence through measures of the HPA axis that self-medication in the βE-KOs with alcohol did not effectively lower CRH levels in stress-related regions of the brain. A lack of increased alcohol consumption in response to stressful states could either indicate resilience against the stressor, or an inability to effectively mitigate the aversive state. In β-endorphin depleted animals, we suspect that the latter factor would drive an absence of exercise restriction-induced alcohol consumption.

In βE-HT animals, castration significantly lowered running behavior compared to shams of the same genotype, as well as compared to βE-KO shams. There is a trend for castration to reduce running in this way for each genotype and with additional animals, we hypothesize that there will be an overall effect of gonadal condition, wherein castration results in significantly lowered activity. This would be consistent with results that we have seen in our other gonadectomy studies, as well as with literature that suggests general decreases in physical activity following gonadectomy (i.e. Richter, 1993). Previous experiments in this data suggest that because drinking patterns do not covary with running patterns, the differences in running behavior between conditions likely do not reflect differences in the saliency of the running wheel.

Should the results of this experiment show a significant interaction between β-endorphin and testosterone, a follow-up study would ideally involve including testosterone-replaced animals to recover the phenotype of the intact animals. Doing so would demonstrate that the effect was mediated by testosterone, rather than other factors that may be involved with being gonadally intact. Since our investigation was exploratory and we did not know what effect of castration would occur in the β-endorphin deficient

animals, we did not include such a replacement group. As in the experiment with the β-endorphin deficient females, this study would also be strengthened by replication with opioid receptor antagonists, which would further strengthen the observations from the knockout study and would provide further information about the mechanism of β-endorphin's action in this paradigm.

General Discussion

Our investigation of gonadal hormones and β-endorphin in alcohol consumption induced by exercise restriction revealed minimal effects of ovarian and testicular hormones and modest effects of β-endorphin in females. Therefore, previous findings that females elevate their alcohol consumption and preference significantly in response to exercise restriction while males do not likely cannot be explained by activational effects of gonadal hormones. In females, lowered β-endorphin may confer elevate susceptibility to drinking in response to exercise restriction, although the successful self-medication of the aversive state may be dependent upon the ability to release β-endorphin with sufficiently high alcoholism that it would inhibit the stress response. In the context of our study, this pattern could be seen in the βE-HTs, where high levels of alcohol consumption may have negatively regulated the CRH expression in the PFC, whereas βE-KOs did not appear to display such negative regulation of the stress response through alcohol consumption. Elevated CORT levels in βE-HTs indicate an elevated experience of stress, perhaps increasing the motivation to self-medicate a state of negative emotionality with alcohol consumption. It is important to note that CORT and CRH act widely throughout the body and brain and the results that we have presented do not suggest isolated action in the PVN of the hypothalamus. Although not investigated here, there remains a strong possibility that gonadal hormones and β-endorphin play important organizational roles in brain structure and function relating to susceptibility to drinking in response to an aversive stimulus, such as the restriction of access to exercise.

There was a general tendency throughout each experiment for all groups to increase their consumption across the experimental period. It is possible that four days of baseline drinking was not a sufficient amount of time to completely acquire individual self-acquisition behavior. In future studies, extending the baseline period long enough for each animal to reach stable daily consumption may be sufficient to remove this effect of time, or would clarify that the escalation in consumption over time that we observed was not entirely explained by the acquisition period. We also did not test our animals for dependence at the conclusion of our study. While we aimed to investigate the effects of exercise restriction on acute consumption, it is possible that some animals may have become dependent to some degree by the end of the 14-day alcohol exposure period. Some models of the transition into alcohol dependence demonstrate that dependence can begin to develop after two weeks of alcohol exposure, and is accompanied by noted changes in brain structures and behavior (Carnicella, Ron, & Barak, 2014; Griffin, 2014; Vendruscolo & Roberts, 2014). Because we did not measure withdrawal symptoms, we cannot comment on what aspects of these data may or may not be applicable to dependent drinkers.

The first aim of these studies was to elucidate the activational roles of gonadal hormones in creating sex differences in exercise restriction-induced alcohol consumption. Sex differences in aversive stimulus-related alcohol consumption have been observed specifically in models of exercise restriction (Piza-Palma et al., 2014), but also in more general and thoroughly validated models of stress (Agaibo et al., 2016; Rivier, 1993), primarily demonstrating that females are more susceptible to elevate their consumption in

such circumstances. In order to evaluate the activational effects of gonadal hormones in this sex difference, we gonadectomized adult male and female mice prior to voluntary exercise and intermittent ethanol exposure. There appear to be little to no activational influences of either ovarian or testicular hormones in exercise restriction-induced consumption increases. Ovarian hormones may encourage elevated levels of alcohol consumption, but do not appear to increase susceptibility to exercise restriction-induced alcohol consumption; females are equally vulnerable to increasing alcohol consumption in response to exercise restriction when ovarian hormones are present and when they are removed. Castration did not significantly change overall drinking or exercise restriction-induced changes in alcohol consumption, although there was a mild tendency for castration to increase overall consumption. One possible explanation for these results is the fact that many local actions of androgens, particularly in the brain, follow local conversion to estrogens (Malyala, Kelly, & Rønnekleiv, 2005). Therefore, estrogen-mediated actions of testosterone may persist even when testosterone has largely been removed. Our results suggest the possibility that gonadal hormones work together to produce the sex-difference seen in overall drinking patterns, where females tend to drink more alcohol than males. Ovarian hormones may promote higher levels of alcohol consumption, concurrent with testicular hormones promoting lower levels of alcohol consumption. However, neither appear to have a notable effect on sex-driven differences in susceptibility to exercise restriction-induced alcohol consumption.

It is important to note that while gonadal steroid hormones are primarily released from the gonads, estrogen and testosterone can also be synthesized locally or produced de

novo in the periphery and the brain. It is therefore possible that gonadal hormones do indeed have activational effects that lead to these sex differences and that the gonadal source of these hormones is simply not entirely responsible for mediating those effects. A valuable follow-up study would be to administer estrogen and androgen receptor antagonists in order to mimic a complete depletion of gonadal hormones. A similar approach could be used to study possible organizational effects of gonadal hormones if the receptor antagonists or biosynthetic enzyme inhibitors were administered during development.

In addition to exploring the role of gonadal hormones in alcohol consumption following exercise restriction, we investigated the role of β-endorphin levels in the risk to engage in alcohol consumption following exercise restriction. β-endorphin appears to play a different role in males and females. Whether β-endorphin is directly interacting with gonadal hormones differently is impossible for us to conclude because we did not do a study in females parallel to experiment 3, but certainly in susceptibility to exercise restriction-induced alcohol consumption there is a notable difference. While in females lacking β-endorphin, whether partially or entirely, increased exercise restriction-induced drinking, a complete absence of β-endorphin in males completely eliminated elevation of alcohol consumption in response to running wheel restriction. A future study of the interaction between ovarian hormones and β-endorphin would provide a more comprehensive picture of how β-endorphin and gonadal hormones may work together to produce sex differences in alcohol consumption patterns. Additionally, while β-endorphin levels in females altered CORT and CRH production, we did not examine such changes

in our study of β-endorphin's interaction with testosterone. Perhaps the related elevated activity of the HPA axis in βE-HT mice and the suggested dysregulation of the HPA axis in βE-KO mice would persist in the male β-endorphin model or there could be different HPA regulation in males that could explain β-endorphin effects. Our lab is continuing the expand the sample sizes in the study for β-endorphin and testicular hormone interactions to increase power for detecting significant relationships.

While knock-outs models are incredibly useful for evaluating the necessity of factors to behavior, constitutive models do not accurately model clinically relevant cases, where people may have lowered levels of a hormone without completely lacking it. βE-HT mice, who do only have lowered levels of β-endorphin, are particularly valuable for the purpose of this clinical relevance. Clinical data has shown a correlation between depressed levels of β-endorphin and elevated risk for excessive drinking, so use of an animal model that can represent this state is helpful for understanding the mechanisms behind that observed clinical correlation (Froehlich et al., 1990; Gianoulakis, 2009). Knock-out animals sometimes display global developmental compensation, where biochemical pathways that may have used β-endorphin develop in an altered fashion to maintain functionality. For this reason, knock-out studies are most effectively complemented with selective receptor agonists or antagonists, which allows for specific manipulation of targeted pathways. Because β-endorphin acts at μ, κ, and δ opioid receptors this would likely require a long investigative process, but using selective opioid receptor agonists or antagonists could further mechanistic understanding of β-endorphin actions.

We were surprised to see that sham surgery seemed to alter behavior in both males and females. Sham animals are included in order to control for possible effects of a surgical procedure, so that comparison with the manipulated group is solely on the basis of the targeted manipulation. The fact that differences between naïve and ovariectomized groups were the only significant findings for females, for instance, could be confounded by the fact that naïve animals did not have surgery. In an attempt to explain what it was about the surgery that was causing sham behavior to differ enough from naïve behavior to not be significantly different from ovariectomized animals, we conducted a preliminary study including animals who received anesthesia (100mg/kg ketamine, 12mg/kg xylazine), but did not go through any further stages of the gonadectomy surgery. There were no conclusive findings from the addition of this group (data not shown), leaving the effect of sham surgery unknown. However, we included this anesthesia group because of emerging research regarding long-term effects of single-dose ketamine administration (i.e., Sabino, Narayan, Zeric, Steardo, & Cottone, 2013).

Conclusions

The overall results of the work presented in this thesis suggest that activational effects of gonadal hormones do not play a significant role in alcohol consumption sex differences following exercise restriction, although ovarian hormones appear to promote elevated average alcohol consumption. β-endorphin deficiency in females also may confer an increased susceptibility to exercise restriction-induced alcohol consumption, but there appears to be no interaction between β-endorphin and testicular hormones in male subjects. Together, these results encourage that levels of gonadal hormones present at a given time may not impact the risk of engaging in alcohol consumption during experience of an aversive stimulus, modeled here by restriction of the appetitive running wheel stimulus. However, β-endorphin deficiency may confer elevated risk to consume alcohol in such circumstances and may have clinical validity in the use of alcohol to self-medicate states of negative emotionality. Understanding the mechanisms through which gonadal hormones and β-endorphin influence alcohol consumption in response to an aversive state may facilitate detection of risk factors in a clinical setting and assist in the ultimate development of individualized treatment options for individuals with AUDs. Research on female alcohol consumption and related sex differences such as that presented here addresses a historic lack of research on females in neuroscience (Beery & Zucker, 2011). Continuing to expand the knowledge of female experience with alcohol will facilitate the development of targeted treatment and prevention options for women, who are making up an increasing proportion of alcohol abusers (Greenfield et al., 2010).

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